Studies on the vitamin K-dependent carboxylase in bovine liver : properties, purification procedure and a possible reaction mechanism

STUDIES ON THE VITAMIN K-DEPENDENT CARBOXYLASE IN BOVINE LIVER: PROPERTIES, PURIFICATION PROCEDURE AND A POSSIBLE REACTION MECHANISM

PROEFSCHRIFT

ter verkrijging van de graad van Doctor in de Geneeskunde aan de Rijksuniversiteit Limburg te Maastricht op gezag van de Rector Magnificus Prof. Dr. H.C. Hemker, hoogleraar, volgens het besluit van het College van Dekanen in het openbaar te verdedigen in de Aula van de Universiteit op vrijdag 18 juni 1982 des namiddags te 16.00 uur

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aan mijn ouders

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CHAPTER 1

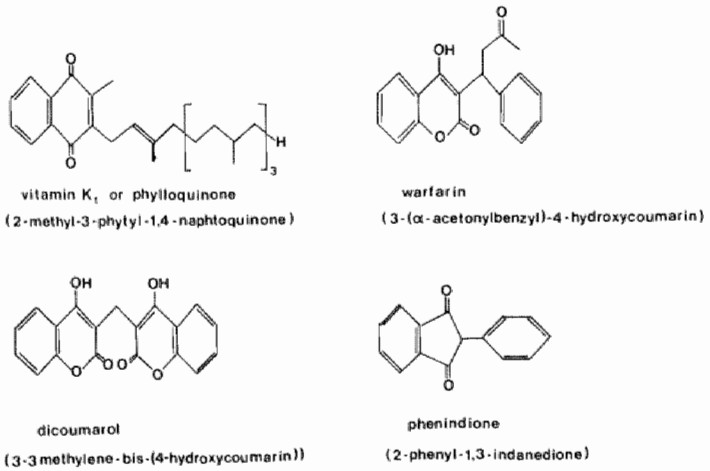
INTRODUCTION

1.1. VITAMIN K AND THE FORMATION OF γ-CARBOXYGLUTAMIC ACID.

Vitamin K and vitamin K antagonists: historical background. When studying the effects of a cholesterol free diet on chicks in the 1930s, Dam noticed these animals developed a hemorrhagic diathesis. This hemostatic defect was related to a deficiency of the fat soluble vitamin K (1), which resulted in a decrease of the prothrombin activity (2). Until the 1950s it was assumed that the only effect of a vitamin K deficiency was a decreased plasma prothrombin level. Nowadays it has been accepted that vitamin K functions in the biosynthesis of the clotting factors II, X, VII and IX (3), the plasma proteins C (4), Z (5) and S (6) and of numerous other proteins in various organs as kidney (7), bone (8), placenta (9) and in chick chorio-allantoic membranes (10). The early work on vitamin K has been reviewed by several autors (11,12).

Since 1921 a hemorrhagic disease of cattle was known in the U.S.A. and Canada. This bleeding sympton was found to be caused by feeding improperly cured sweet clover hay. The coumarin derivates in the hay, discovered by

Fig. 1. Structures of vitamin K_1 and some vitamin K antagonists.



Campbell and Link (13,14), are antagonizing the action of vitamin K. Subsequently a large number of substituted 4-hydroxycoumarins have been synthesized and their anticoagulant action is most helpful as a rodenticide and in the clinic for the control of thrombogenic episodes. The clinical use of these 4-hydroxycoumarins has been reviewed by O'Reilly (15) and their structure-function relationships by Renk and Stoll (16). The anticoagulant action of 2-substituted-1,3-indanediones, which appear to act all in the same way as the coumarins, was recognized by Kabat et al (17). Only the coumarin drugs are recommended for therapy as more frequent side-effects are caused by indanediones. The structures of vitamin K, some coumarin derivates and phenindione are depicted in fig. 1.

Vitamin K and precursor proteins. The first who suggested that a prothrombin precursor was involved in the vitamin K-dependent synthesis of prothrombin were Hemker et al. (18). They noticed that the plasma of dicoumarol treated patients contained an abnormal form of prothrombin, which they called PIVKA II (protein induced by vitamin K absence). This finding was confirmed immunologically by Ganrot and Nilehn (19) and Josso et al.(20). Abnormal forms of clotting factors X, IX and VII have also been detected in the plasma of anticoagulated humans (21-29).

In the plasma of anticoagulated cows abnormal forms of prothrombin, factor X and factor IX have been detected (30,31). Bovine abnormal prothrombin (32-34) and abnormal factor X (35) have been purified. They both lack biological activity, but can be activated non-physiologically. Abnormal prothrombin can generate thrombin with the aid of trypsin (33), Echis carinatus venom (33) or staphylocoagulase (36,37). After activation with Russel's viper venom abnormal factor X has amidolytic activity towards Bz-Ile-Glu-Gly-Arg-p-nitroaniline (35), but no activity in blood coagulation can be generated from abnormal factor X. In the plasma of other anticoagulated species abnormal clotting factors have not been demonstrated with the exception of some abnormal prothrombin in the plasma of anticoagulated chicks (38), horses, sheep, dogs, guiney pigs, rabbits and goats (C. Vermeer, unpublished observations).

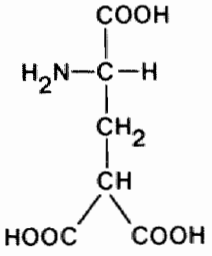
The presence of hepatic prothrombin precursors was demonstrated by Shah and Suttie (39) in the microsomal fraction of rat liver. Thrombin activity could be generated from the precursors by treatment with Echis

Carinatus venom. The amount of hepatic precursors increased after the administration of warfarin or after a vitamin K-deficient diet, but it decreased rapidly after a subsequent injection of vitamin K. As the level of precursor fell, the amount of microsomal normal prothrombin raised until the prothrombin was demonstrated in the plasma. These data indicated that hepatic prothrombin precursors were used for the vitamin K-dependent synthesis of biologically active prothrombin. The first vitamin K-dependent in vitro system producing prothrombin from precursor prothrombin molecules was described by Shah and Suttie in 1974 (40). They used the microsomal fraction of livers of vitamin K-deficient rats.

Several forms of abnormal prothrombin have been isolated from the liver microsomal fraction of warfarin treated rats (41,42) and from cultured rat hepatoma cells (43). They are all immunologically similar to prothrombin and their molecular weights are 72000 d. Only their isoelectric points are mutually different. The purified hepatic abnormal prothrombins have not been shown to produce prothrombin in a cell-free system.

γ-carboxyglutamic acid: a new amino acid. Most studies concerning the vitamin K dependent structures in proteins are done with bovine prothrombin and abnormal prothrombin purified from plasma. Those molecules are indistinguishable with respect to their molecular weight, their amino acid composition after acid hydrolysis and their carbohydrate composition (33,44,45). The most striking difference is the ability of prothrombin to bind calcium ions, which is required for its association with phospholipids, whereas abnormal prothrombin does not bind calcium ions (46,47,48). The two forms of prothrombin are also electrophoretically different in the presence of calcium ions (49). The calcium binding properties were located

Fig. 2. Srtucture of γ - carboxyglutamic acid



in the NH_2-terminal portion of prothrombin (45) and an acidic peptide was isolated from this region, which could not be isolated from abnormal prothrombin (50). Stenflo, reported that a γ-carboxyglutamic acid (gla) residue was present in this peptide (51) and the presence of gla in prothrombin was confirmed by others (52,53). The structure of gla is shown in fig. 2.

Magnusson (54) reported the amino acid sequence of prothrombin and the first 10 glutamic acid residues at the N-terminal end had an extra carboxyl group on the γ-C-atom. This -carboxylation is probably the only vitamin K-dependent modification in prothrombin. The discovery of gla has been reviewed extensively (55,56).

After the demonstration of gla residues in bovine prothrombin numerous other proteins in plasma as well as in several organs have been reported to contain gla (57). Until now the only known physiological significance of the γ-carboxylation is related with the calcium binding properties of proteins with gla residues. This is illustrated by the enhanced activity of the vitamin K-dependent clotting factors in the presence of Ca^{++} and phospholipids. Furthermore vitamin K-dependent proteins might be involved in the calcium mobilization of the egg shell (10,58) and the deposition of calcium in bone matrix (59).

The vitamin K-dependent carboxylation. Esmon et al (60) demonstrated that the rat liver homogenates which were generating prothrombin activity (39), were also catalyzing a vitamin K-dependent carboxylation reaction. Radioactive prothrombin was formed after incubation with ^{14}C labeled bicarbonate and vitamin K. The label was located in the γ-carboxyglutamic acid residues of the NH_2 terminal region. Since then vitamin K-dependent carboxylation has been studied most extensively in microsomal liver preparations of vitamin K-deficient or warfarin-treated rats. The endogenous precursors present in those preparations were used as substrates for the carboxylation reaction. After the development of synthetic substrates (61-63) increasingly more studies are done with those low molecular weight peptides e.g. Phe-Leu-Glu-Glu-Leu.

The vitamin K-dependent carboxylation reaction has been reviewed by several autors (55-57, 64,65) among which the extensive review by Suttie (57). The main properties of the reaction are cited in table 1.

Table 1. Properties of the vitamin K-dependent carboxylase.

Absolute requirements
- vitamin K and NAD(P)H, vitamin K and dithiotreitol, or reduced vitamin K
- O_2
- CO_2
- CO_2 acceptor (endogenous substrate or a synthetic peptide)
Inhibitors
- 2-chloro-3-phytyl-1,4-naphtoquinone (chloro-K)
- 2,3,5,6-tetrachloro-4-pyridinol
- coumarin anticoagulants
- sulphydryl reagents
Stimulators
- dithiotreitol
- pyridoxalphosphate

Effective vitamin K activity appears to be related with 2-methyl-1,4-naphtoquinone with some substitutuents in the 3-position (66,64) and it is generally believed that the reduced form of vitamin K is the active component which mediates carboxylation (67,68). Also the tritium release from Phe-Leu-Glu-Glu-Leu, tritiated at the γ-carbon of each Glu residue, requires the reduced form of vitamin K (69).

The role of oxygen in the carboxylation reaction, which has not been studied in any detail, will be discussed below. The active species of "CO_2" initially participating in the carboxylation reaction is postulated to be CO_2 and not HCO_3^- (70). These data are not conclusive, however, as CO_2 is transported faster across the nonaqueous phase than HCO_3^- and carboxylation occurs in nonaqueous membranes (71,72,73). The possible involvement of a vitamin K hemicarbonate in the carboxylation reaction (74) is not substantiated by experimental investigation (75).

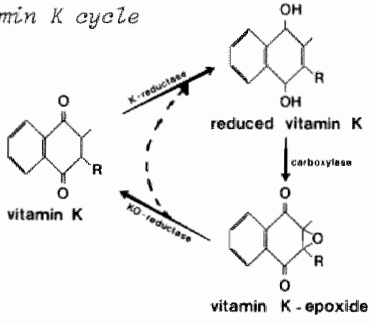
The carboxylase activity, as measured with endogenous substrate, is almost exclusively associated with the luminal membrane surface of rough endoplasmic reticulum (17,73). The smooth endoplasmic reticulum probably contains a small amount of carboxylase activity, which can only be measured with a peptide substrate (73). The carboxylase activity can be extracted

from the membrane with several non-ionic surfactants and bile salts (57,76,77). A substantial purification of those micellar solutions with carboxylating activity has not been published.

The vitamin K-dependent carboxylation in other tissues has not been studied as extensively as in hepatic tissues, but it seems that the requirements for carboxylation are similar in bone microsomes (8), lung microsomes (78) and the chick embryonic chorioallantoic membrane (10).

The relationship between the epoxidation of vitamin K and the vitamin K-dependent carboxylation. Vitamin K is partly present in the form of its 2,3-epoxide in liver (79) and vitamin K "epoxidase" activity has been demonstrated in isolated microsomes (80). The hydroquinone form of vitamin K is converted to the epoxide and the epoxide oxygen is generated by molecular oxygen (81). The epoxide can be reduced again to vitamin K by the vitamin K epoxide reductase (82,83,84) and the reduction of vitamin K quinone can be carried out by NAD(P)H dehydrogenase (85,86), also termed DT-diaphorase (EC 1.6.99.2) and possibly by vitamin K reductase (84) or vitamin K epoxide reductase (87). Thus vitamin K epoxide formed from vitamin K hydroquinone can be converted to vitamin K hydroquinone, which is called the vitamin K cycle (fig. 3). The reduction of the epoxide is inhibited by coumarins and this seems the biologically important action of these anticoagulants (11,55,88). The vitamin K cycle is also active in other tissues, where vitamin K-dependent carboxylation has been demonstrated (86).

Fig. 3. The vitamin K cycle



The biological function of the vitamin K cycle could be the constant generation of reduced vitamin K for the carboxylation reaction. In this concept the carboxylation reaction is in some way coupled to the epoxidation event and several arguments have been put forward to support this hypothesis. Many requirements for the epoxidation and carboxylation reaction are similar (81), the subcellular location of both activities is the same (73) and both activities may have a common hydroperoxide intermediate of vitamin K (90). Furthermore it has been demonstrated that the formation of vitamin K epoxide is elevated in rat livers, which contain high levels of endogenous substrate for the carboxylation reaction (80). Vitamin K epoxide formation could also be increased in microsomal preparations of normal rats by the addition of synthetic substrates for the carboxylase (91). The amount of epoxide formed was related to the amount of carboxylation events, but whenever the number of moles CO_2 fixed per mole epoxide formed is calculated there is more epoxide formed than CO_2 fixed in most cases (91,57). By raising the CO_2 concentration equal amounts of vitamin K epoxide and γ-carboxyglutamic acid can be formed (92). Thus epoxidation can take place without carboxylation. On the other hand carboxylation without expoxidation nas never been demonstrated clearly and the concept that both events are coupled in some way has generally been accepted.

1.2. DECARBOXYLATION AND CARBOXYLATION REACTIONS.

Decarboxylation and carboxylation: general consideration. The mechanisms of carboxylation and decarboxylation reactions will be shortly surveyed here. A plausible mechanism for the vitamin K-dependent carboxylation reaction is not included, but will be discussed in chapter 8. The reaction we are dealing with is

$$RH + CO_2 \rightleftharpoons RCOOH \qquad \textit{(equation 1)}$$

The equilibrium is often closely balanced, but chelation of the carboxylated product e.g. with Mg^{++} may shift the equilibrium in favour of the carboxylation (93).

The enzymes catalyzing carboxylation and/or decarboxylation reactions

can be divided in seven groups according to their content of different coenzymes (table 2). Furthermore one biotin dependent transcarboxylation reaction is known.

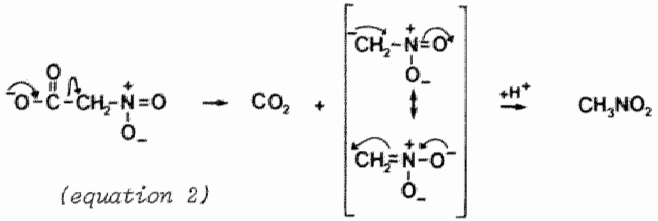
Table 2. The various coenzymes for carboxylases and decarboxylases.

reaction catalyzed	coenzyme
decarboxylations	pyridoxalphosphate thiaminepyrophosphate pyruvate
decarboxylations and carboxylations	biotin ferredoxin none
carboxylations	vitamin K

The reaction mechanisms of the enzyme catalyzed reactions seem not to differ from the mechanisms developped by the organic chemists and specific properties of these reactions will not be discussed. The interested reader is referrred to the comprehensive book of Walsh (94).

Mechanism of decarboxylation reactions. For the decarboxylation of RCOOH both the removal of a hydrogen atom and of carbondioxide from the R-group is necessary. The removal of hydrogen is usually accomplished by having the reaction proceed in a base environment. After the removal of hydrogen and carbondioxide from a carboxylic acid a carbanion remains. It would thus be expected that this mode of decarboxylation is assisted in those organic compounds where the resultant carbanion is stabilized by electronwithdrawing groups, especially when they can exert a mesomeric as well as an inductive effect. Illustrative is the extremely ready decomposition of nitroacetic acid (equation 2).

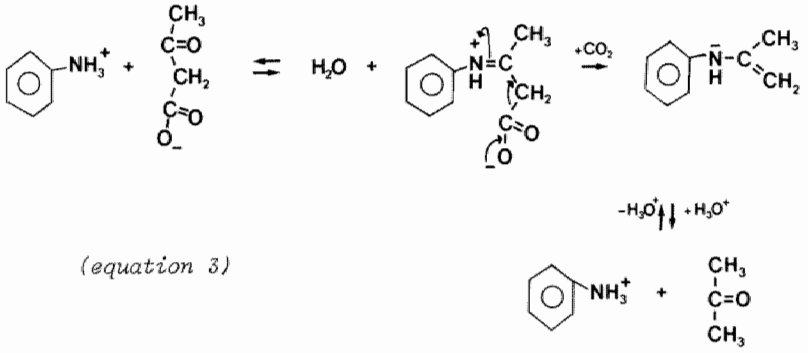
Catalysis of decarboxylation reactions will be effected by stabilizing the carbanion. Divalent metal ions (Cu^{++}, Mn^{++}), strong acids and primary amines are used for this purpose. The role of metal ions in catalysis is due to their electrophilic character, thus stabilizing negative charges. In



(equation 2)

a strong acidic environment the substrate may be protonated and the positive charge after protonation will effectively serve as an "electron-sink" for the negative charge after the release of carbondioxide. The coenzymes pyridoxalphosphate and thiamine pyrophosfate are protonated at neutral pH and exert catalytic activity via this protonation.

Catalysis by primary amines is effected by the formation of a Schiff base from the condensation of the amine with a carboxyl group (equation 3). The Schiff base may be protonated at neutral pH and therefore the principle of catalysis is the same as catalysis by strong acids. An example is the decarboxylation of acetoactetate in the presence of aniline (equation 3).

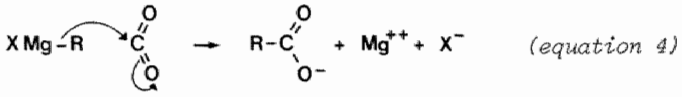


(equation 3)

The catalysis by acetoacetate decarboxylase (95,96) (E.C.4.1.1.4.) resembles the catalysis by aniline. In this case the Schiff base is formed from acetoacetate and a lysine residue of the enzyme; the electronshift during decarboxylation is as depicted in equation 3. The coenzymes pyridoxalphosphate and pyruvate also form a Schiff base with their substrates (amino acids) in order to catalyze the decarboxylation.

Mechanism of carboxylation reactions. The carboxylation reaction as depicted in equation 1 is usuallly quite slow and therefore carboxylation reactions are generally performed under conditions where R exists as a carbanion or has considerably carbanion character.

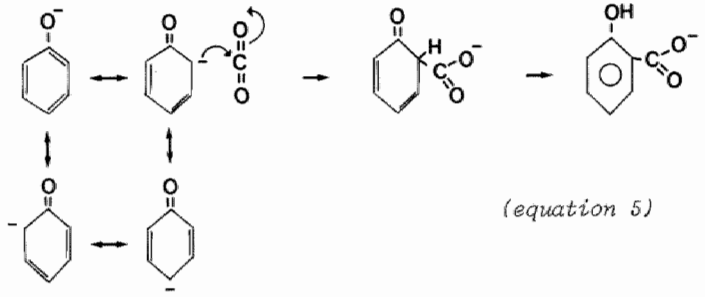
The best known carboxylation reaction is the reaction of carbondioxide with Grignard reagents (equation 4). The actual structure of Grignard reagents is complicated and still a matter of some dispute, but for convenience the general formula of alkylmagnesiumhalides RMgX is used. The magnesiumhalogen bond is essentially ionic. The carbon-magnesium bond is covalent, but also highly polar. In other words, the organic group of Grignard reagents has considerable carbanion character and reacts with numerous inorganic compounds such as carbondioxide.



(equation 4)

Organosodium and organolithium reagents undergo a similar carboxylation reaction as Grignard reagents.

The preparation of o-hydroxybenzoic acid (salycylic acid) from dry sodium phenoxide with carbon dioxide is a classical example of a Kolbé-Schmidt reaction. The mechanism appears to involve attack of carbon dioxide at the activated ortho position of the phenoxide ion (equation 5)



(equation 5)

In this reaction some p-hydroxybenzoic acid is formed as well, which may be expected from the resonance structures of the phenoxide ion.

Essential for the carboxylation of Grignard reagents or for the Kolbé-Schmidt reaction is the formation of a negatively charged carbon

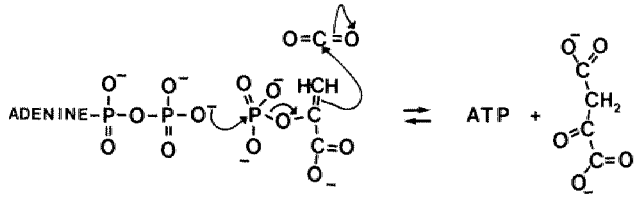
atom, which can be attacked by the electrophilic carbon of carbondioxide. The carbanion can also be formed by the reduction of radicals. Although this process is not as common as the formation of carbonium ions by the oxidation of radicals (97), radicals with electron withdrawing groups e.g. α-ketoalkylradicals are reduced relatively readily.

For the carboxylation of a radical without its prior reduction, the formation of a carbondioxide anionradical is required. After that step a radical-radical combination reaction can occur (equation 6).

$$R\bullet + \bullet CO_2^- \rightarrow RCOO^- \qquad \textit{(equation 6)}$$

The carbondioxide anionradical is experimentally formed during electrolytic reduction of carbon dioxide to formic acid and oxalic acid (98,99) or probably via an electrontransfer from an anionradical of 1,4-dicyanobenzene and 9,10-dicyanoanthracene to carbondioxide in a polar medium (100).

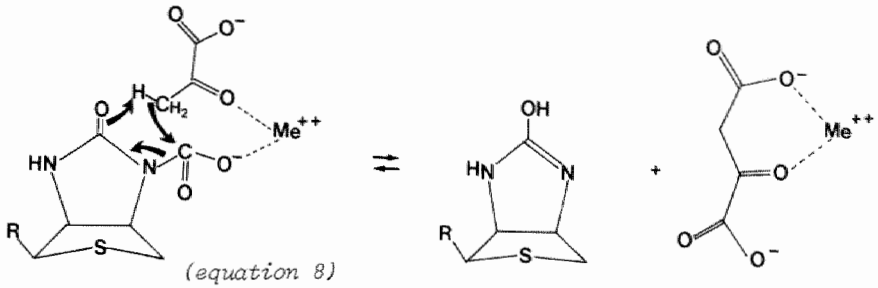
The principle of the formation of a negatively charged carbon atom before addition of carbondioxide is applicable to some enzyme catalyzed carboxylations as well e.g. the carboxylation of phosphoenolpyruvate by phosphoenolpyruvatecarboxylase (E.C. 4.1.1.49) (equation 7).



(equation 7)

The mechanism consists of a nucleophilic attack on the phosphorylgroup of phosphoenolpyruvate, initiating an electron shift of the double bond and addition of carbon dioxide at the β-carbon of phosphoenolpyruvate.

The reaction catalyzed by biotin enzymes proceeds in two steps. The first step is the formation of the key intermediate 1-N-d-carboxybiotin and the second step is the carboxyltransfer from N-carboxybiotin to an acceptor molecule. For the carboxyltransfer to pyruvate in the presence of Mn^{++}, Co^{++} or Zn^{++} the following mechanism is proposed (equation 8) (101).



(equation 8)

An analogous mechanism is postulated for the carboxyltransfer to coenzyme A containing substrates or to water in decarboxylation reaction.

Ferredoxin-dependent carboxylation reactions are exclusively found in photosynthetic and anaerobically fermentative bacteria. The carboxylation reaction is coupled to the oxydation of reduced ferredoxin and the electrons are possibly transported from ferredoxin to the substrate by endogenous iron-sulphur chromophores (94).

1.3. INTRODUCTION TO THIS THESIS

Vitamin K functions in the γ-carboxylation of glutamic acid residues, which takes place in various tissues and organs. The importance of these γ-carboxyglutamic acid-residues (their Ca^{++} binding properties), is demonstrated by the process of blood coagulation. Administration of vitamin K antagonists, which blocks the formation of gla e.g. in the clotting factors II, X, IX and VII has a strong anticoagulant effect. Therefore vitamin K antagonists, especially the 4-hydroxycoumarin derivates, are most popular in antithrombotic therapy. As thrombosis and its results are a major cause of death in the Netherlands, the importance of controling thrombogenic eposides can hardly be exaggerated.

Until now the properties of enzymes catalyzing the vitamin K-dependent reaction, are not known in any detail and chemical data about the γ-carboxylation of glutamic acid residues, catalyzed by vitamin K, are not available. Hence the action of vitamin K and vitamin K antagonists is poorly understood. In this thesis some aspects of the vitamin K-dependent

carboxylase from bovine liver and a purification procedure for the enzyme are described. A mechanism for the vitamin K-dependent carboxylation will be postulated.

REFERENCES

1. Dam, H. (1935) Biochem. J. 29, 1273
2. Dam, H. Schönheyder, F. and Tage-Hansen, E. (1936) Biochem. J. 30, 1075
3. Davie, E.W. and Fujikawa, K. (1975) Ann.Rev.Biochem. 44, 799
4. Stenflo, J. (1976) J.Biol.Chem. 251, 355
5. Prowse, C.V. and Esnouf, M.P. (1977) Biochem.Soc.Trans. 5, 255
6. Discipio, R.G., Hermondson, H.A., Yates, S.G. and Davie, E.W. (1977) Biochem.J. 16, 698
7. Hauschka, P.V., Friedman, P.A., Traverso, H.P. and Gallop, P.H. (1976) Biochem.Biophys.Res.Commun. 71, 1207
8. Lian, J.B. and Friedman, P.A. (1978) J.Biol.Chem. 253, 6623
9. Friedman, P.A., Hauschka, P.V., Shia, M.A. and Wallace, J.K. (1979) Biochim.Biophys.Acta 583, 261
10. Tuan, R. (1979) J.Biol.Chem. 254, 1336
11. Suttie, J.W. (1978) in Fat Soluble Vitamins, Handbook of Lipid Research Vol. 2 (De Luca, H.F. ed.), Plenum Press, New York, 211.
12. Almquist, H.J. (1975). Am.J.Clin.Nutr. 28, 656
13. Campbell, H.A. and Link, K.P. (1941) J.Biol.Chem. 138, 21
14. Link, K.P. (1959) Circulation 19, 97
15. O'Reilly, R.A. (1976) Ann.Rev.Med. 27, 245
16. Renk, E. and Stoll, W.G. (1968) Prog.Drug.Res. 11, 226
17. Kabat, H., Stohlman, E.F. & Smith, M.J. (1944) J. Pharmacol.Exp.Ther. 80, 160
18. Hemker, H.C., Veltkamp, J.J., Hensen, H. and Loeliger, E.A. (1963). Nature 200, 589
19. Ganrot, P.O. and Nilehn, J.E. (1968) Scand.J.Clin.Lab.Invest. 22, 23
20. Josso, F., Lavergne, J.M. and Soulier, J.P. (1970) Nouv.Res.Fr. Hematol., 633

21. Denson, K.W.E. (1971) Brit.J.Haemat. 20, 643
22. Goodnight, S.H., Feinstein, D.I. Østerud, B. and Rapaport, S.I. (1971) Blood 38, 1
23. Levanon, M., Rimon, S., Shari, M., Ramot, B. and Golberg, E. (1972) Brit,J.Haematol. 23, 669
24. Brozovic, M. and Howarth, D.J. In "Prothrombin and related coagulation factors " Proceedings of a Boerhaave Course (Hemker, H.C. and Veltkamp, J.J. Eds.), Leiden University Press , Leiden, 252-263
25. Larrieu, M.S. and Meyer, O. (1970) Lancet 11, 1085
26. Veltkamp, J.J., Muis, H., Muller, A.D., Hemker, H.C. and Loeliger, E.A. (1971) Thrombos.Diathes.Haemorrh. 25, 312
27. Lechner,K. (1972) Thrombos. Diathes. Haemorrh. 27, 19
28. Gaudernack, G., Gladhaug, B.A., Østerud, B. and Prydz, H. (1974) Thrombos.Diathes.Haemorrh. 31, 40
29. Gaudernack, G. and Prydz, H. (1975) Thromb.Diathes.Haemorrh. 34, 455
30. Reekers, P.P.M., Lindhout, M.J., Kop-Klaassen, B.M.M. and Hemker, H.C. (1973) Biochim.Biophys.Acta 317, 559
31. Lindhout, M.J., Kop-Klaassen, B.H.M. and Hemker, H.C. (1976) J.Mol.Med. 1, 223
32. Malhotra, O.M. and Carter, J.R. (1971) J.Biol.Chem. 246, 2665
33. Nelsestuen, G.L. and Suttie, J.W. (1972) J.Biol.Chem. 247, 8176
34. Stenflo, J. and Ganrot, P.O. (1972) J.Biol.Chem. 247, 8160
35. Lindhout, M.J., Kop-Klaassen, B.H.M., Kop, J.M.M. and Hemker, H.C. (1978) Biochim.Biophys.Acta 533, 302
36. Josso, F., Lavergne, J.M., Gouault, M., Prou-Wartelle, O. and Soulier, J.P. (1968) Thromb.Diathes.Haemorrh. 20, 88
37. Bas, B.M., Muller, A.D. and Hemker, H.C. (1974) Biochim.Biophys.Acta 379, 164
38. Carlisle, T.L., Shah, D.V., Schlegel, R. and Suttie, J.W. (1975) Proc.Soc.Exp.Biol.Med. 148, 140
39. Shah, D.V. and Suttie, J.W.(1971) Proc.Natl.Acad.Sci. U.S.A. 68, 1653
40. Shah, D.V. and Suttie, J.W. (1974) J.Biol.Chem. 250, 4744
41. Grant, G.A. and Suttie, J.W. (1974) Biochemistry 15, 5387
42. Grant, G.A. and Suttie, J.W. (1976) Arch.Biochem.Biophys. 176, 650
43. Graves, C.B. Grabau, G.G. Olson, R.E. and Munns, T.W. (1980) Biochemistry 19, 266

lo, J. (1972) J.Biol.Chem. 247, 8167
lo, J. (1973) J.Biol.Chem. 248, 6325
stuen, G.L. and Suttie, J.W. (1972) Biochemistry 11, 4961
lo, J. and Ganrot, P.O. (1973) Biochim.Biophys.Res.Commun. 50, 98
, J. and Stenflo, J. (1973) FEBS Lett. 32, 343
lo, J. (1970) Acta Chem.Scand. 24, 3762
stuen, G.L. and Suttie, J.W. (1973) Proc.Natl.Acad. Sci. U.S.A.
366
lo, J., Fernlund, P., Egan, W. and Roepstorff, P. (1974)
Natl.Acad.Sci. U.S.A. 71, 2730
stuen, G.L., Zytkovicz, T.H. and Howard, J.B. (1974) J.Biol.Chem.
6347
sson, S., Sottrup-Jensen, L., Petersen, T.E., Morris, H.R. and
A. (1974) FEBS Lett. 44, 189
sson, S., Sottrup-Jensen, L., Petersen, T.E. and Claeys,H. (1975)
Prothrombin and Related Coagulation Factors" Proceedings of a
laave course, (Hemker, H.C. and Veltkamp, J.J. eds.) Leiden
rsity Press, Leiden, 25
on, C.M. and Suttie, J.W. (1977) Progress in haematology 10, 333
lo, J. and Suttie, J.W. (1977) Ann.Rev.Biochem. 46, 157
e, J.W. (1980) C.R.C. Crit.Rev.Biochem. 8, 191
R. (1980) in "Vitamin K metabolsim and Vitamin K-dependent
ins (Suttie, J.W. ed) University Park Press, Baltimore, 294
p, P.M., Lian, J.B. and Hauschka, P.V. (1980) The New Eng.J.Med.
1460
, C.T., Sadowski, J.A. and Suttie, J.W. (1975) J.Biol.Chem. 250,

, J.W., Hageman, J.M., Leherman, S.R. and Rich, D.H. (1976)
l.Chem. 251, 6827
r, R.M., Carey, D.J., Bus, K.M., Marshall, G.R. and Olson, R.E.
) FEBS Lett. 75, 226
e, J.W., Lehrman, S.R., Geweke, L.O., Hageman, J.M. and Rich, D.H.
) Biochem.Biophys.Res.Commun. 86, 500
on, B.C. (1980), in Methods in Enzymology 67, 165
e, J.W., Canfield, L.M. and Shah, D.V. (1980) in Methods in
ology 67, 180

66. Mack, D.O., Wolfsenberger, M., Girardot, J.M., Miller, J. and Johnson, B.C. (1979). J.Biol.Chem. 254, 2656
67. Sadowski, J.A., Esmon, C.T. and Suttie, J.W. (1976) J.Biol.Chem. 253, 2770
68. Friedman, P.A. and Shia, M. (1976) Biochem.Biophys.Res.Commun. 70, 647
69. Friedman, P.A., Shia, M.A., Gallop, P.M. and Griep, A.E. (1979), Proc.Natl.Acad.Sci. U.S.A. 76, 3126
70. Jones, J.P. Gardner, E.J. Cooper, T.G. and Olson, R.E. (1977) J.Biol.Chem. 252, 7738
71. Helgeland, L. (1977) Biochim.Biophys.Acta 499, 181
72. Wallin, R. and Prydz, H. (1979) Thromb. Haemostasis 41, 529
73. Carlisle, T.L. and Suttie, J.W. (1980) Biochemistry 19, 1161
74. Friedman,P.A. and Shia, M.A. (1977) Biochem. J. 163, 39
75. Chander, K.S., Gaudry, M., Marquet, A., Rikong-Adie, H., Decottignies-Lemarechal, P. and Azerad,R. (1981) Biochim. Biophys. Acta 673, 157
76. Esmon, C.T. and Suttie, J.W. (1976) J.Biol.Chem. 251, 6238
77. Mack, D.O., Suen, E.T., Girardot, J.M., Miller, J.A., Delaney, R. and Johnson, B.C. (1976) J.Biol.Chem. 251, 3269
78. Bell, R.G. (1980) Arch.Biochem.Biophys. 203, 58
79. Matschiner, J.T., Bell, R.G., Amelotti, J.M. and Knauer, T.E. (1970) Biochim.Biophys.Acta 201, 309
80. Willingham, A.K. and Matschinen, J.T. (1974) Biochem.J 140, 435
81. Sadowski, J.A., Schnoes, H.K. and Suttie, J.W. (1977) Biochemistry 16, 3856
82. Zimmerman, A. and Matschiner, J.T. (1974) Biochem.Pharmacol. 23, 1033
83. Matschiner, J.T., Zimmerman, A. and Bell, R.G. (1974) Thromb.Diath. Haemorrh.Suppl. 57, 45
84. Whitlon, D.S., Sadowski, J.A. and Suttie, J.W. (1978) Biochemistry 17, 1371
85. Martius, C., Ganser, R. and Viviani, A. (1975) FEBS Lett. 59, 13
86. Wallin, R., Gebhardt, O and Prydz, H. (1978) Biochem.J. 169, 95
87. Sherman, P.A. and Sander,E.G. (1981) Biochem. Biophys. Res. Commun. 103, 997
88. Suttie, J.W. (1977) Semin.Haemotol. 14, 365
89. Bell, R.G. (1978) Fed. Proceed. 37, 2599
90. Larson,A.E., and Suttie,J.W. (1978) Proc.Natl.Acad.Sci U.S.A. 75, 5403

91. Suttie, J.W., Geweke, L.O., Martin, S.L. and Willingham, A.K. (1980) FEBS Lett. 109, 267
92. Larson, A.E., Friedman, P.A. and Suttie, J.W. (1981) J.Biol.Chem. 256, 11032
93. Stiles, M. and Finkbeiner, H.L. (1959) J.Am.Chem.Soc. 81, 505
94. Walsh,C. (1979) Enzymatic Reaction Mechanisms, W.H. Freeman and Company, San Fransisco, chapter 21 and chapter 22
95. Everse, J. Barnett, R.E., Thorne, J.R. and Kaplan, N.O. (1971) Arch.Biochem.Biophys. 143, 444
96. Coulson, C.J. and Rabin, B.R. (1969) FEBS Lett. 3, 333
97. Nonhebel, D.C., Tedder, J.M. and Walton, J.C. (1979) in Radicals, Cambridge University Press, Cambridge, 138
98. Jordan, J. and Smith, P.T. (1960) Proc.Chem.Soc. 246
99. Ryu, J., Anderson, J.N. and Eyring, H.J. (1972) J.Phys.Chem. 76, 3278
100. Tazuke, S. and Kitamura, N. (1978) Nature 275, 301
101. Visser, C.M. (1977) Ph.D. Thesis, Rijksuniversiteit Groningen, Groningen

CHAPTER 2

A COMPARISION BETWEEN VITAMIN K-DEPENDENT CARBOXYLASE FROM NORMAL AND WARFARIN-TREATED COWS.

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SUMMARY

Detergent-solubilized microsomal preparations that are catalyzing the vitamin K-dependent γ-carboxylation of glutamic acid residues in peptide and protein substrates, have been obtained from the livers of normal and warfarin-treated cows. The preparations from warfarin-treated animals contained more endogenous substrate than those from normal cows, but otherwise the two preparations were indistinguishable. The enzymes vitamin K reductase and γ-glutamyl carboxylase may function independently of each other in this system. They are, nevertheless, intimately linked in some way, so that the reduced vitamin K that is produced by the former enzyme can be used immediately by the latter.

INTRODUCTION

Vitamin K-dependent carboxylase has been obtained from the livers of various animals. Administration of warfarin to the animals leads to a considerable increase in the amount of endogenous substrate (1), and most studies dealing with the vitamin K-dependent carboxylase have utilized the endogenous substrate for the reaction (2). The endogenous substrate, which accumulates in cow liver after the administration of warfarin, consists mainly of precursors of clotting factor X, and it co-purifies with the enzyme (chapter 4). In this chapter, the properties of carboxylase preparations from normal and warfarin-treated cows have been compared in order to see if both types of carboxylase are similar, and wether conclusions obtained with the carboxylating enzyme system from normal animals can thus also be applied to that from warfarin-treated animals and vice versa.

MATERIALS AND METHODS

Chemicals. Vitamin K was obtained from Hoffmann-La Roche (Basel, Switzerland) and vitamin K hydroquinome was prepared as described previously (3). Vitamin K-epoxide was prepared according to Fieser et al.

(4). The synthetic substrate Phe-Leu-Glu-Glu-Leu was obtained from Vega Fox (Tucson, USA) and $NaH^{14}CO_3$ (40 Ci/mol) from New England Nuclear (Dreieich, GFR). Glucose oxidase, Triton X-100, warfarin and dithiothreitol (DTT) were purchased by Sigma (Saint Louis, USA) Picofluor-15 by Packard Instruments (Warrenville, USA). All other chemicals were obtained from Merck (Darmstadt,GFR).

Preparation of carboxylase. One year old calves were anticoagulated by the oral administration of warfarin (10 mg/kg daily) during one week. Crude microsomes were prepared from the livers of normal and warfarin-treated cows as described earlier (3), and carboxylase was solublilized from the microsomal pellet by adding buffer containing 0.8 M KCl, 0.05 M Tris-HCl (pH 7.5), 0.5% Triton X-100 and 30% ethylene glycol. The obtained solution with a final protein concentration of 50 mg/ml was used for all experiments described below.

Measurement of carboxylase activity. Unless indicated otherwise, the vitamin K-dependent incorporation of $^{14}CO_2$ was measured by incubating soluble carboxylase (5 mg protein) and 20 µCi $NaH^{14}CO_3$ in 0.25-ml reaction mixture containing 0.4 M KCl, 0.05 M Tris-HCl (pH 7.5), 10 mM DTT, 12% ethylene glycol, 0.2% Triton X-100 and 10 mM Phe-Leu-Glu-Glu-Leu. The reaction was started by adding 0.2 mM vitamin K hydroquinone and the mixtures were incubated in parafilm-sealed tubes at 25°C. The reaction was stopped with 2 ml 10% trichloroacetic acid and the precipitates (containing the carboxylated endogenous substrate) were washed and counted in a Packard Tricarb scintillation counter using Picofluor-15 scintillation liquid. The trichloroacetic acid-supernatants (containing the carboxylated Phe-Leu--Glu-Glu-Leu) were degassed at 80°C and counted.

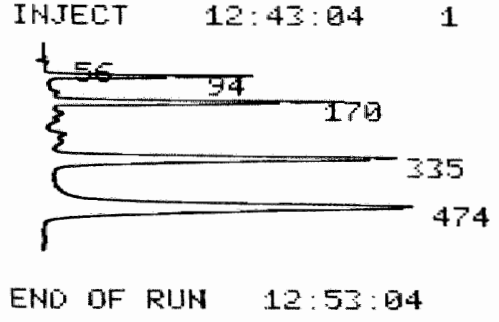
When the carboxylation reaction was performed in the absence of oxygen, the incubations occurred in Warburg-flasks. Before the reaction was started, 0.4 ml of a mixture containing glucose (0.1 M) and glucose oxidase (0.5 mg) in 0.1 M NaCl and 0.05 M Tris-HCl, pH 7.4 was applied in the centre well, vitamin K in the side-arm vessel and the other components of the reaction mixture in the outer well. The bulk of the oxygen was removed by flushing with nitrogen gas, the flasks were closed and the last traces of oxygen were removed by incubating the flasks for 1 h at 25°C. After this

period the carboxylation reaction was started by adding the vitamin K hydroquinone to the reaction mixture in the outer well.

Statistical analyses. The significances of the differences between the means of the results for the normal and the warfarin-treated groups (four cows in each group) were analyzed by a one-tailed Wilcoxon rank-sum test.

Determination of vitamin K hydroquinone, vitamin K and vitamin K-epoxide. The various forms of vitamin K were extracted from the 1 ml reaction mixtures, by adding 10 ml hexane and 15 ml isopropanol and shaking the mixtures for 5 min. After this period, 5 ml water was added and the upper phase was removed. The solvent was evaporated and the remaining vitamin K was dissolved in 0.5 ml methanol and analyzed by means of high-performance liquid chromatography (HPLC) on a Lichrosorb 10 RP 18 column (Chrompack, 25x0.46 cm) in methanol. This technique was performed on a Spectrophysics SP 8000 liquid chromatograph with a flow rate of 2 ml/min. The three forms of vitamin K were well separated when the effluent was monitored at 254 nm (fig 1). The area under each peak was recorded automatically and was corrected for the relative absorbance at the detection wavelength.

Fig. 1. Separation profiles of vitamin K (474 s), vitamin K hydroquinone (170 s) and vitamin K epoxide (335 s).



The various forms of vitamin K (1 µg of each) were seperated and detected by their absorbance at 254 nm. Calibration curves of each of the components were prepared at this wavelenght and used for the determination of the absolute amounts of the various forms of vitamin K.

RESULTS

We compared the properties of the vitamin K-dependent carboxylase present in 8 different solubilized microsomal preparations. Four preparations were obtained from normal cows and the four other preparations from cows that had received warfarin. We first investigated the optimal conditions for the carboxylation reaction. They were equal for both types of carboxylase, and were as follows: 25°C, pH 7.5, 0.01 M DTT, 0.3-0.7 M KCl and 0.5 % Triton X-100. The K_m values for vitamin K hydroquinone, CO_2 and Phe-Leu-Glu-Glu-Leu were determined and found to be equal for both groups (0.05, 0.17 and 11.5 mM, respectively). For technical reasons, we were unable to estimate and compare the K_m for O_2 of the different preparations. Both types of carboxylase were completely comparable,however, in their dependece upon O_2: in the absence of oxygen the incorporation of $^{14}CO_2$ amounted 109 $\pm$ 8 dpm per h per mg protein and in the presence of oxygen 1472 $\pm$ 103 dpm (means of 8 experiments $\pm$ S.E.).

The enzyme activities were compared by measuring the incorporation of $^{14}CO_2$ into Phe-Leu-Glu-Glu-Leu. The Phe-Leu-Glu-Glu-Leu concentration was 10 mM because at concentrations greater than 20 mM a marked substrate inhibition of the carboxylation was observed. With 10 mM Phe-Leu-Glu-Glu-Leu and 0.2 mM vitamin K hydroquinone, the carboxylation rate, after a possible lag phase to be discussed later, was constant for at least 1 h and and it was proportional to the amount of enzyme preparation added. When we compared the enzyme activity per mg protein of the different preparations under these conditions, no significant difference between the groups was found: the carboxylation rate in the preparations obtained from normal cows was 30.0 + 3.2 dpm per min per mg protein and in preparations from the four warfarin-treated animals the rate was 35.2 $\pm$ 4.2 dpm per min per mg protein.

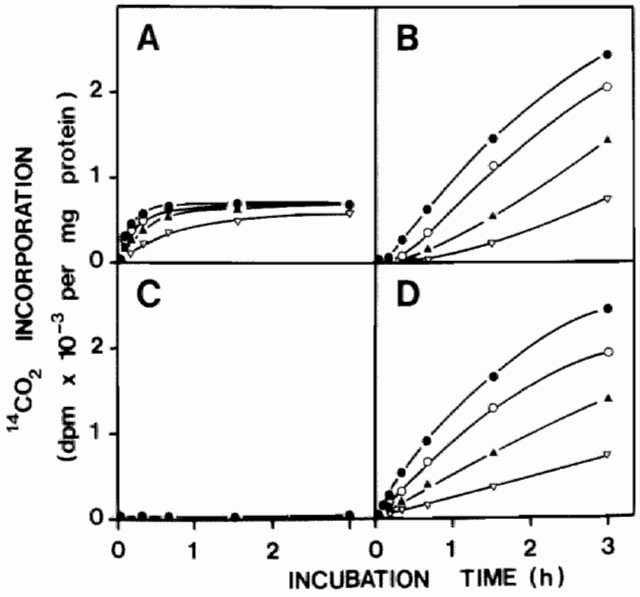
When CO_2 incorporation was determined in the absence of exogenous substrate, activity was observed only in carboxylase preparations from warfarin-treated animals (fig. 2A, C). This incorporation is interpreted as carboxylation of endogenous substrate. The final level of incorporated CO_2 is proportional to the amount of the enzyme preparation added, and this level is a measure for the amount of endogenous substrate contained in this preparation. Carboxylase preparations from warfarin-treated cows contain

significantly more endogenous substrate than the carboxylase preparations from normal cows (normal: 23.8 ± 1.6 dpm per mg protein, warfarin-treated: 900 ± 72 dpm per mg protein).

The initial rate of incorporation can be changed by varying amount of vitamin K hydroquinone. This was also found when the carboxylation of exogenous substrate was measured (fig. 2 B,D). The K_m values for vitamin K hydroquinone estimated from the initial rate of CO_2 incorporation in endogenous and exogenous substrate, were equal. The data in fig. 2 show that, only when endogenous substrate is present, a lag time occurs before the carboxylation of exogenous substrate starts. This is readily explained by a preferential carboxylation of the endogenous substrate.

We also investigated whether oxidized forms of vitamin K could be used for the carboxylation reaction. (fig. 3)

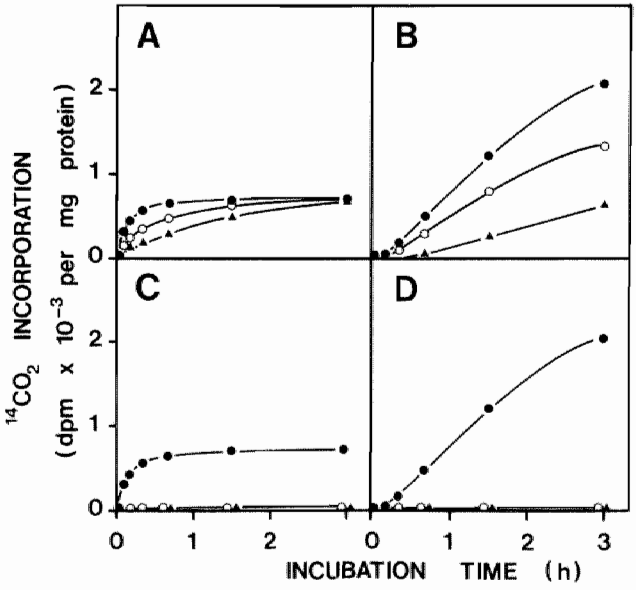
Fig. 2. Time course of the carboxylation at various vitamin K concentrations.



The carboxylation was performed at 0.2 mM (●——●), 0.1 mM (○——○), 0.05 mM (▲——▲) and 0.025 mM (▽——▽) vitamin K hydroquinone. Panel A: carboxylase from warfarin-treated cows, endogenous substrate. Panel B: carboxylase from warfarin-treated cows, exogenous substrate. Panel C: carboxylase from normal cows, endogenous substrate. Panel D: carboxylase from normal cows, exogenous substrate.

As shown in fig. 3, both endogenous and exogenous substrates can be carboxylated in the presence of DTT, when vitamin K or vitamin K epoxide were used instead of vitamin K hydroquinone. This carboxylation was completely blocked by 20 μM warfarin. In the rat, this level of warfarin is known to strongly inhibit the reductase (2). At equal concentrations of the coenzymes, the oxidized forms lead to lower carboxylation rates than that resulting from the reduced form. This indicates that the reduction step is rate-limiting. When carboxylase preparations from normal cows (which do not contain endogenous substrate) are preincubated with oxidized forms of vitamin K and DTT, no carboxylation can occur because of the lack of substrate, and a pool of reduced vitamin K is formed, and this was demonstrated directly. Preincubated reaction mixtures were extracted with

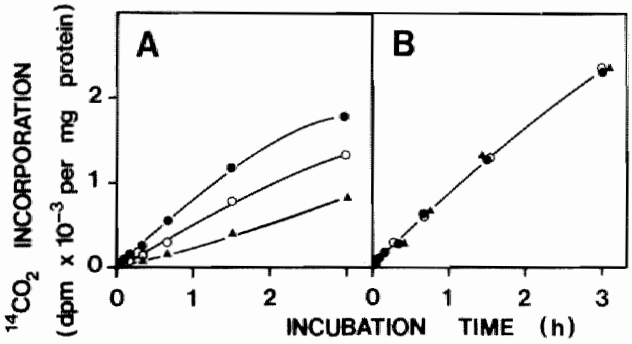
Fig. 3. The effect of various forms of vitamin K on carboxylase from warfarin-treated cows.



The carboxylation was performed at 0.4 mM vitamin K hydroquinone (●——●), vitamin K (○——○)or vitamin K epoxide (▲——▲). Panel A: endogenous substrat Panel B: exogenous substrate. Panel C:endogenous substrate plus 20 μM warfarin. Panel D: exogenous substrate plus 20 μM warfarin.

isoproponal/hexane and the extracts were analyzed for their vitamin K hydroquinone content using HPLC. After 1 h in the presence of vitamin K, 21 nmol vitamin K hydroquinone had been formed in the 1-ml reaction mixtures. Under similar conditions, vitamin K epoxide was partly reduced to 14 nmol vitamin K and 5 nmol vitamin K hydroquinone per ml reaction mixture after 1 h at 25°C. When, after this preincubation period, the carboxylation reaction was started by addition of Phe-Leu-Glu-Glu-Leu, the carboxylation reaction proceeded at the maximal rate for all three forms of vitamin K (fig. 4).

Fig. 4. The effect of various forms of vitamin K on carboxylase from normal cows.



The carboxylation was performed at 0.4 mM vitamin K hydroquinone (●——●), vitamin K (○——○) or vitamin K epoxide (▲——▲). Panel A: without preincubation. Panel B: after 1 h preincubation at 25 °C in the absence of Phe-Leu-Glu-Glu-Leu and $^{14}CO_2$.

DISCUSSION

Upon comparison of the properties of the vitamin K-dependent carboxylase in microsomal preparations of normal and warfarin treated cows, it can be concluded that the only demonstrable difference is in the amount of endogenous substrate present. The reasons are: 1. The optimal reaction

conditions for both types of preparation were equal; 2. The K_m values for vitamin K hydroquinone, CO_2 and exogenous substrate were equal, and both enzyme systems were similarly dependent of O_2; 3. The amount of enzyme activity per mg protein, measured by carboxylation of exogenous substrate under standard conditions, were not significantly different; 4. The amount of CO_2 incorporation in the absence of exogenous substrate in carboxylase preparations from the warfarin-treated cows was about 40 times higher than in carboxylase preparations from normal cows.

The cow is dissimilar to the rat in this respect, as it has been shown by Shah and Suttie (5) and confirmed by us (unpublished data), that in rat liver the amount of carboxylase increases during warfarin treatment, whereas in cow-liver this treatment did not affect the enzyme level. As is known from the rat system (6-8), vitamin K hydroquinone is required for the carboxylation reaction. When vitamin K or vitamin K epoxide is used, the vitamin must be reduced by its reductase before the carboxylation reaction can proceed. With the endogenous, and also exogenous substrate, the reduction of vitamin K is the rate-limiting step, because the reduced form will always cause a substantially higher activity, if the carboxylase activities obtained with equal amounts of oxidized and reduced forms are compared.

The carboxylase can function independently of the reductase, with both exogenous and endogenous substrates, because 20 µM warfarin (which inhibits the reductase completely) will not inhibit the carboxylation if vitamin K hydroquinone is present. On the other hand, the reductase can function independently of the carboxylase, because vitamin K and its epoxide will still be reduced when no substrate for carboxylation is present (carboxylase preparations from normal cows, tested without exogenous substrate). If substrate is addded after preincubation with oxidized forms of vitamin K, the carboxylation proceeds as if vitamin K hydroquinone was present. The amount of vitamin K hydroquinone that were formed amounted to 10-50% of the K_m value. Nevertheless, the carboxylation proceeded at its maximal reaction rate. This indicates that the newly-formed vitamin K hydroquinone is not equally distributed within the microsomal membranes, but remains in high local concentrations in the environment of the carboxylase/reductase. Therefore, these two enzymes cannot be distributed over the microsomal membrane remnants independent of each other, but have to be linked in such

a way, that the vitamin K hydroquinone produced by the reductase is channeled preferentially to the carboxylase.

In our experiments, we have not yet been able to differentiate between the vitamin K-reducing enzyme and that reducing vitamin K-epoxide. Whether this means that both functions are fulfilled by the same enzyme, or enzyme system remains to be investigated.

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REFERENCES

1. Carlisle, T.L., Shah, D.V. and Suttie, J.W. (1980) in Vitamin K metabolism and vitamin K-dependent proteins (Suttie, J.W., ed), pp 443-449, University Park Press, Baltimore
2. Suttie, J.W. (1980) C.R.C. Crit.Rev.Biochem. 8, 191-223
3. Vermeer, C., Soute, B.A.M. and Hemker H.C. (1978) Biochim.Biophys.Acta 523, 494-505
4. Fieser, L.F., Tishler, M. and Sampson, W.K. (1941) J. Biol. Chem. 137, 659-692
5. Shah, D.V. and Suttie, J.W. (1978). Arch. Biochem. Biophys. 191, 571-577
6. Sadowski, J.A., Schnoes, H.K. and Suttie, J.W. (1977) Biochemistry 16, 3856-3863
7. Friedman, P.A. and Shia, M. (1976) Biochem. Biophys. Res. Comm. 70, 655-662
8. Girardot, J.M., Mack, D.O., Floyd, R.A. and Johnson, B.C. (1976) Biochem. Biophys. Res. Comm. 70, 65-662

CHAPTER 3

PREPARATION OF AN ACID-SOLUBLE SUBSTRATE FOR VITAMIN K-DEPENDENT CARBOXYLASE BY LIMITED PROTEOLYSIS OF BOVINE DESCARBOXYPROTHROMBIN.

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SUMMARY

Bovine descarboxyprothrombin and descarboxyfragement-1 can be used as substrates for rat and bovine vitamin K-dependent carboxylase. In both enzyme systems, however, these substrates have a high K_m (0.3-0.4 mM). A better substrate (K_m = 0.001-0.003 mM) was prepared from bovine descarboxyprothrombin by limited a proteolysis with subtilisin Carlsberg. This substrate is called Fragment-Su and is composed of the amino acids 13-29 of descarboxyprothrombin.

INTRODUCTION

The vitamin K-dependent carboxylation reaction is generally studied with rat carboxylase prepared from warfarin-treated or vitamin K-deficient animals. Precursor proteins, which are substrate molecules for the vitamin K-dependent carboxylase are present in those preparations and it has been demonstrated that prothrombin is synthesized from the precursors (1.2). It could however not be demonstrated with those preparations that purified hepatic prothrombin precursors were carboxylated (3).

During the last few years we have developed a cow liver cell-free system in which purified descarboxyporthrombin is converted into prothrombin, as measured with a coagulation assay (4,5). The enzyme system was designated as prothrombin synthase and not as carboxylase, because parallel incorporation of $^{14}CO_2$ into descarboxyprothrombin could not unequivocally be demonstrated. This system is therefore unsuitable for measuring the carboxylation of various substrates and we preferred to use solubilized microsomes from the liver of normal cows for these studies. As has been pointed out in chapter 2 these microsomes hardly contain endogenous substrate for the vitamin K-dependent carboxylase, whereas the carboxylating activity towards exogenous substrates can be measured easily. In this chapter we describe the preparation of a low molecular weight fragment from bovine descarboxyprothrombin that may be used as a trichloroacetic acid-soluble substrate for both rat and bovine carboxylase.

Chemicals and buffers. Buffer A: 0.15 M NaCl/0.01 M Tris-HCl, pH 7.5. Vitamin K_1 was obtained from Hoffmann-La Roche (Switzerland). Triton X-100 and dithiotreitol (DTT) were from Sigma (USA). Proteases were obtained from Sigma (USA) and Boehringer (FRG). $NaH^{14}CO_3$ (60 mCi/mmol) was purchased from the Radiochemical Centre Amersham (UK) and Aquasol-2 was from New England Nuclear (USA). Vitamin K hydroquinone was prepared as described in (4). The synthetic pentapeptide Phe-Leu-Glu-Glu-Leu was obtained from Vega Fox (USA). DEAE-Sephacel and CNBr-activated Sepharose were obtained from Pharmacia (Sweden) and proteins were coupled to the Sepharose according to the manufacturers prescription. Ultrogel was obtained from LKB (Sweden). All other chemicals were obtained from Merck (FRG).

Preparation of carboxylase. Soluble bovine carboxylase was prepared from the liver of non-anticoagulated cows. The crude microsomes, prepared as described in (4) were suspended in a buffer containing 0.8 M NaCl, 0.05 M Tris-HCl (pH 7.5), 1 mM DTT, 0.5 mM EDTA, 0.5% Triton X-100 and 30% ethylene glycol. The final protein concentration was 50 mg/ml and insoluble material was removed by centrifugation (140.000 x g for 1.5 h). The supernatant of this run was designated as soluble carboxylase. Sepharose-bound carboxylase was prepared as described in chapter 4. Soluble rat carboxylase was obtained from anticoagulated female rats (200-220 g) of the Lewis strain. The rats were anticoagulated by the intraperitoneal adminstration of 2 mg sodium warfarin in 0.9% NaCl 18 h before being killed. The animals were killed by decapitation and the livers were removed, washed with 0.25 M sucrose and used for the preparation of soluble carboxylase in a similar way as that described for the bovine system.

Measurement of carboxylase activity. Reaction mixtures (0.25 ml) containing 0.1 ml soluble carboxylase (3.5 mg protein), 0.3 M NaCL, 0.05 M Tris-HCl, pH 7.5, 0,2% Triton X-100, 12% ethylene glycol, 20 µCi $NaH^{14}CO_3$, 2 mM dithiothreitol, 100 µM vitamin K hydroquinone and exogenous substrate as indicated were incubated at 25°C in parafilm-sealed tubes. The reaction was stopped at various intervals by diluting the samples with 2 ml ice-cold buffer A and 2 ml 10% trichloroacetic acid. After centrifugation, traces of

unbound label were removed from the supernatant by vacuum extraction and the samples were counted in Aquasol-2 in a Packard Tricarb scintillation counter. The trichloroacetic acid-precipitates were dissolved in NaOH and reprecipitated three times before counting. The results are expressed as dpm per mg of protein.

Preparation of purified substrates. Bovine dicoumarol plasma (4) was adsorbed with $BaSO_4$ (200 g/l) and descarboxyprothrombin was purified therefrom by chromatography on QAE-Sephadex, DEAE-Sephadex, hydroxylapatite and Sepahdex G-100. Bovine thrombin was obtained from Hoffmann-La Roche and was purified by chromatography on SP-Sephadex and benzamidin-agarose. Descarboxyfragment-1 was prepared by incubating purified descarboxyprothrombin and thrombin in a molar ratio of 10: 1 in buffer A at 37°C for 2 h. Descarboxyfragment-1 was resolved from the other components of the digest by chromatography on Sephadex G-100 in 20% acetic acid as described by Stenflo (6) and dialysed against buffer A.

Amino acid analysis, NH_2-terminal sequence and end group determinations. Amino acid analysis was performed after hydrolysis in 6 M HCl in vacuo at 110°C for 20 h using a Beckman 119 CL amino acid analyzer. NH_2-terminal amino acid sequence analysis was performed as described by Edman (7). In some instance μ^{14}Cß-phenylisiothiocyanate was used. Identification of the phenylthiohydantoins was performed by thin-layer chomatography (8). Radioactive derivates were identified with a Berthold scanner and quantitated by liquid scintillation counting. COOH-terminal amino acids were determined by digestion with carboxypeptidase Y (Pierce) and amino acid analysis (9). About 80 nmol of protein was dissolved in 0.05 M sodium acetate buffer, pH 5.5, to a protein concentration of about 1.6 mg/ml. Digestion was performed at 25°C using an enzyme/substrate ratio of 1 : 250 (w/w) and sampling over a time period of 5-90 min. The sample was kept in a boiling water bath for 3 min to inactivate the enzyme and diluted with 0.2 M sodium citrate buffer, pH 2.2, for analysis.

RESULTS

When we tried to carboxylate exogenous substrates in a bovine carboxylating enzyme system, it resulted that at a concentration of 1.4 M (plasma concentration) purified bovine descarboxyprothrombin did not affect the carboxylation reaction (Table I) Descarboxyfactor X also did not affect the carboxylation reaction at plasma concentration (0.28 μM). Only at more than 70-fold higher concentrations of descarboxyprothrombin a significant increase of $^{14}CO_2$ incorporation was measured. In order to exclude the possibility that descarboxyprothrombin merely stimulates carboxylase without being carboxylated itself, we modified either the endogenous substrate or the exogenous one in such a way that they could easily be separated from each other after the carboxylation reaction had occurred. The three modification procedures that we used were: (a) preparing descarboxyfragment-1, which, in contrast to the endogenous substrate, is soluble in thrichlroacetic acid. (b) Cross-linking the descarboxyprothrombin to CNBr-activated Sepharose and (c) preparing Sepharose-bound carboxylase; in this partly purified system all endogenous substrate is linked to the solid phase and can thus be separated from the soluble exogenous descarboxyprothrombin (5,chapter 4).

Table I. The carboxylation of various substrates by rat and bovine carboxylase

Mixtures containing rat and bovine carboxylase were incubated at 25 °C for 0.5 and 1 h, respectively. The carboxylation by bovine carboxylase is presented in *italics*.

Substrate added	Vitamin K-dependent incorporation of $^{14}CO_2$ in			
	Trichloroacetic acid precipitate		Trichloroacetic acid supernatant	
None	252	*68*	1	*1*
Descarboxyprothrombin (1.4 μM)	245	*70*	1	*1*
Descarboxyprothrombin (0.1 mM)	454	*345*	2	*2*
Descarboxyfragment-1 (1.4 μM)	261	*65*	3	*5*
Descarboxyfragment-1 (0.1 mM)	232	*47*	206	*183*

When using descarboxyfragment-1 we observed that this substrate enhanced the carboxylation reaction in a similar way as descarboxyprothrombin did. Since the additional amount of incorporated label was present in the trichloroacetic acid-soluble fraction (Table I) it is obvious that descarboxyfragment-1 is carboxylated by the rat as well as by the bovine enzyme system. It resulted that rat carboxylase was also able to carboxylate the solid-phase descarboxyprothrombin (Sp-DP) in a vitamin K-dependent way (Table II). In the bovine system, however, it appeared that

Table II. The carboxylation of bovine descarboxyprothrombin by rat and bovine carboxylase

The concentration of exogenous substrate (Sp-DP) was 0.2 ml slurry (0.9 mg of protein) per ml reaction mixture. The incorporation of $^{14}CO_2$ into Sp-DP was measured after 1 h at 25 °C by filtering the reaction mixtures over glass-fiber filters, followed by extensive washing with buffer A. The filters were counted in Aquasol and the results are expressed as dpm per mg of protein. The concentration of solid-phase carboxylase (Sp-carboxylase) was 0.2 ml slurry (0.05 mg of protein) per ml reaction mixture. The incorporation of $^{14}CO_2$ into the soluble substrate was measured after 4 h at 25 °C by filtering the reaction mixtures over glass-fiber filters and counting the filtrate.

Sepharose-bound substrate	$^{14}CO_2$ incorporation by soluble rat carboxylase into Sepharose-bound protein	
A	+vitamin K hydroquinone	-vitamin K hydroquinone
None	7	7
Descarboxyprothrombin	116	20
Prothrombin	14	12
Soluble substrate	**$^{14}CO_2$ incorporation by Sepharose-bound bovine carboxylase into soluble protein**	
B	+vitamin K hydroquinone	-vitamin K hydroquinone
None	1370	982
Descarboxyprothrombin (0.1 mM)	127000	1032
Prothrombin (0.1 mM)	3186	1866

the cross-linking procedure had changed the descarboxyprothrombin in such a way that the Sp-DP did not stimulate the carboxylation reaction any more and neither was it carboxylated itself. On the other hand, when the enzyme and its endogenous substrate were attached to the solid phase, soluble dexcarboxyprothrombin could be added to the enzyme system and it could be demonstrated that also in this case the exogenous substrate was carboxylated (Table II).

The carboxylation of descarboxyprothrombin and descarboxyfragment-1 occurred with a very low efficiency, however, (see below) and we tried to prepare better substrates by degrading descarboxyprothrombin with Sepharose-bound proteases. Sixteeen proteases were used and the effect of their fragmentation products on the $^{14}CO_2$ incorporation was measured in rat as well as in bovine carboxylase (Table III). By keeping the digested prothrombin at a concentration as low as 2.8 µM we could rapidly screen the result of the limited proteolysis, because only strongly improved preparations possessed a demonstrable activity in the carboxylation reaction. From these experiments it became clear that four proteases (subtilisin Carlsberg, subtilisin BPN', pronase P and proteinase K) were able to cleave a highly active fragment from descarboxyprothrombin. In all these cases the carboxylated reaction products were found in the tricholoroacetic acid supernatant, and when assayed on Ultrogel columns, all four substrates had a molecular weight which was lower than 5,000 d. We decided to purify and characterize the substrate that is obtained by the digestion of descarboxyprothrombin with Sepharose-bound subtilisin Carlsberg. This fragment was designated as Fragment-Su and it was purified from the digest by chromatography on DEAE-Sephacel, AcA 54 and Ultrogel A 202 columns (Fig.1).

The final purification was performed by HPLC-chromatography (Fig.2). The carboxylatable peptide was recovered in peak C. The amino acid composition of the purified peptide is summarized in Table IV and is in agreement with the composition that is expected to be found from the amino acid residues 13-29 of descarboxyprothrombin (Fig.3.).

Fig. 3. Amino acids 13-29 of descarboxyprothrombin.

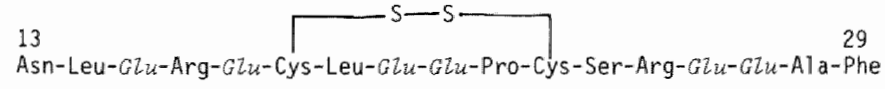
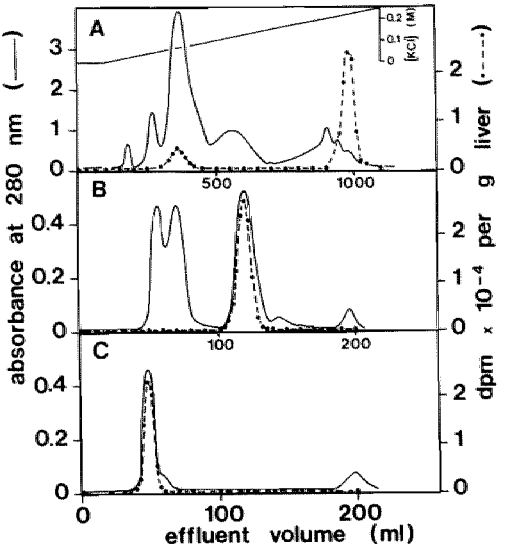


Table III. The carboxylation of fragmented bovine descarboxyprothrombin by rat and bovine carboxylase-

The various proteases were coupled to CNBr-activated Sepharose and 1 ml slurry was incubated with 2.5 mg descarboxyprothrombin for 2 h at room temperature, while rotating end-over-end. The reaction conditions during digestion were: 0.1 M NaCl, 0.02 M Na-acetate buffer, pH 6.0 in the case of pepsin and 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.2 for all other proteases. During the carboxylation reaction the concentration of fragmented substrate (if added) was 200 µg/ml. The carboxylation by rat carboxylase and bovine carboxylase (*italics*) was measured after 60 min at 25 °C; blank values (without vitamin K) were substracted.

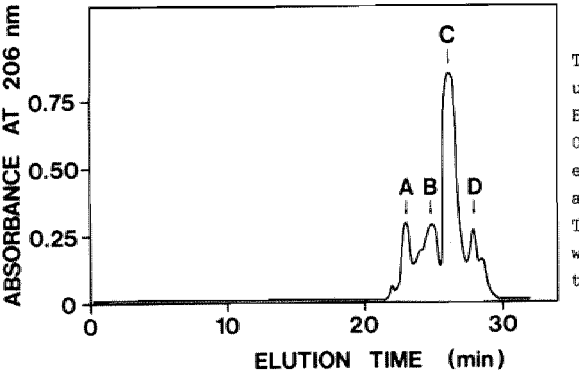
Protease used for the fragmentation of prothrombin	Vitamin K-dependent incorporation of $^{14}CO_2$ in			
	Trichloroacetic acid precipitate		Trichloroacetic acid supernatant	
None	270	*66*	1	*2*
Trypsin	335	*62*	5	*15*
Chymotrypsin	198	*63*	6	*38*
Pepsin	270	*65*	2	*4*
Papain	292	*87*	4	*17*
Subtilisin Carlsberg	219	*37*	37	*158*
Subtilisin BPN'	229	*45*	24	*170*
Protease II	279	*63*	5	*28*
Protease IV	290	*69*	4	*33*
Pronase P	216	*40*	11	*98*
Pronase A	280	*56*	4	*21*
Clostripaine	253	*75*	2	*9*
Proteinase K	229	*44*	39	*212*
Thermolysin	272	*55*	0	*32*
No substrate added	266	*68*	3	*2*

Fig. 1. Fractionation of digested descarboxyprothrombin.



Descarboxyprothrombin (7 µM) was digested with Sepharose-bound subtilisin (10 ml slurry) in a reaction mixture of 600 ml, containing 10 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.2. The digest was adsorbed onto a DEAE-Sephacel column (1.5 × 30 cm) in the same buffer and eluted with a linear gradient (2 × 500 ml) from 10 to 250 mM KCl (panel A). The active fractions were pooled as indicated, concentrated and applied to an AcA 54 column (1.5 100 cm) in 1 mM EDTA, 0.5 M KCl an 10 mM Tris-HCl, pH 7.8 (panel B). The active fractions were pooled and concentrated and applied to an Ultrogel A 202 column (1.5 × 100 cm) in distilled water (panel C). After these fractionations the recovery of carboxylatable material was 37%.

Fig. 2. Purification of Fragment-Su by HPLC-chromatography.



The protein solution was adsorbed to an ultrasphere-ODS (5 µM) column (25 × 1 cm, Beckman) and a gradient was applied from 0-30% CH_3CN in 0.1% H_3PO_4. The peaks were eluted with a flow rate of 4 ml per min and assayed as described in the text. The recovery of purified Fragment-Su was 0.26 mol per mol descarboxyprothrombin.

Table IV. Amino acid composition of fragment -Su.

The figures in the first column are given as mol amino acid per mol of peptide. The second column represents the composition expected from the sequence 13-29 of descarboxyprothrombin.

Amino acid	Composition observed	Composition expected
Asp	1.1	1
Thr	0	0
Ser	0.8	1
Glu	6.2	6
Pro	1.3	1
Gly	0.2	0
Ala	1.0	1
Val	0.1	0
Met	0	0
Ile	0	0
Leu	2.0	2
Tyr	0.1	0
Phe	1.0	1
His	0	0
Lys	0	0
Arg	2.0	2.0

Table V. The kinetic constants of various substrates for rat and bovine carboxylase.

The K_m and V were calculated from the initial rates at various concentrations of the substrates. The V is expressed in dpm incorporated per min per mg of protein. Further details are as described in Materials and Methods.

Substrate	Bovine carboxylase		Rat carboxylase	
	K_m (mM)	V (dpm)	K_m (mM)	V (dpm)
Descarboxyprothrombin	0.4	54	0.3	123
Descarboxyfragment-1	0.4	74	0.3	105
Fragment-Su	0.001	174	0.003	150
Phe-Leu-Glu-Glu-Leu	11	108	8	95

Quantitative amino acid analysis revealed that Asn was the N-terminal residue with a yield of 0.86 mol per mol peptide. The sequence of the amino acid residues at the N-terminal side was found to be: Asn-Leu-Glu-Arg and at the C-terminal position we found Phe in 0.93 mol per mol of peptide. This confirmed that Fragment-Su is identical to the amino acid sequence 13-29 in descarboxyprothrombin.

In order to demonstrate that the incorporated label in the carboxylated Fragment-Su was present in gla-residues, we prepared a carboxylating reaction mixture (1 ml) containing 10 µg of the peptide fragment. After 1 h at 25°C the proteins were precipitated with trichloroacetic acid and non-bound $^{14}CO_2$ was removed (see Materials and Methods). The trichloroacetic acid supernatant contained carboxylated Fragment-Su (98,512 dpm). After acid hydrolysis in 6 M HCl in vacuo at 110°C for 20 h about 50% of the label (51326 dpm) had disappeared, showing that the gla-residues had been decarboxylated into glu. Finally, we compared the kinetic constants of descarboxyprothrombin, descarboxy-fragment-1, Fragment-Su and the synthetic pentapeptide Phe-Leu-Glu-Glu-Leu (Table V). The K_m of Fragment-Su was 0.001 mM, whereas the K_m of the other three substrates ranged from 0.4 to 11 mM. Comparison of the maxiamal carboxylation rates (V) showed that these were all of the same order of magnitude. Therefore, it has to be concluded that Fragment-Su is a better substrate than any of the other exogenous substrates.

DISCUSSION

When bovine descarboxyprothrombin and descarboxyfragment-1 were added to a rat liver vitamin K-dependent carboxylase, both substrates were carboxylated. This could be demonstrated by separating the ^{14}C-labeled exogenous substrates from the reaction mixtures. These experiments exclude the possibility that the added substrates act by promoting the conversion of endogenous substrates.

In bovine liver carboxylase the presence of descarboxyprothrombin and descarboxyfragment-1 induced an increase of the $^{14}CO_2$ incorporation which was similar to that in the rat system. Because descarboxyfragment-1 is not precipitated with trichloroacetic acid, it is separated from the endogenous

substrates in the reaction mixtures and it could thus be shown that also in this case the exogenous substrate was carboxylated. Insolubilized substrates, however, had lost their ability to stimulate the carboxylation reaction. On the other hand, when we used Sepharose-bound carboxylase and soluble descarboxyprothrombin, it could be demonstrated that bovine carboxylase, as well as rat carboxylase, is able to carboxylate this substrate.

Both in the rat and in the bovine system we observed high K_m values for descarboxyprothrombin and for descarboxyfragment-1. This means that high concentrations (0.5-1mM) of these substrates have to be added to carboxylase in order to be able to measure their carboxylation. It is not probable that the endogenous substrates are present in our carboxylase preparations in these quantities and therefore the endogenous substrates must be far better substrates than the exogenous ones. Possible reasons for this phenomenon might be: (a) The main part of the endogenous substrates does not consist of prothrombin precursors and prothrombin precursors are carboxylated rather poorly. (b) Descarboxyprothrombin from plasma differs from liver prothrombin precursors (e.g. because the latter contain a signal-peptide* (10) at the N-terminal side of the molecule). (c) In vivo prothrombin precursors are carboxylated during protein synthesis. After completion of the peptide chain sterical hindrance hampers the carboxylation reaction. (d) In vivo the carboxylation of prothrombin precursors is followed by a second modification (e.g. glycosylation). This second modification occurs independent of the carboxylation reaction and hampers the carboxylation of descarboxyprothrombin.

Since it is known that at least 25% of endogenous reaction product of rat and bovine carboxylase consists of prothrombin (1,2,chapter 4), the possibility mentioned under (a) will not be considered. Furthermore it cannot be excluded that the signal-peptides mentioned under (b) have an effect on the in vitro carboxylation, but direct investigations are hampered by the lack of experimental possibilities. If however, the structure of the completed descarboxyprothrombin hinders the carboxylation reaction (c) and/or (d), it should be possible to remove the main part of the descarboxyprothrombin molecule in order to obtain a substrate that would be more accessible to carboxylase. The removal of fragment-2 and thrombin (resulting in descarboxyfragment-1) did not change the kinetic

characteristics of the endogenous substrate. Only proteolytic degradation of the latter to a peptide containing the amino acids 13-29 resulted in a substantial improvement of the substrate. It is intriguing that the structure of this peptide contains all information which is required for a good substrate, whereas this information is lost in a number of smaller synthetic peptides (11). It may be expected therefore, that further proteolytic cleavage of Fragment-Su will worsen this substrate although it is still to be investigated whether all its 17 amino acids are required for a good carboxylation reaction.

* Many secreted proteins are synthesized as precursors having an NH_2-terminal extension. This extension (10-30 amino acids long) is very rich in hydrophobic residues and has been termed the signal peptide.

REFERENCES

1. Esmon, C.T., Sadowski, J.A. and Suttie, J.W. (1975) J.Biol.Chem. 250,4744-4748
2. Graves, C.B., Grabau, G.G., Olson, R.E. and Munns, T.W. (1980) Biochemistry 19,266-272
3. Suttie, J.W. (1980) C.R.C. Crit.Rev.Biochem. 8,191-223
4. Vermeer, C., Soute, B.A.M. and Hemker, H.C. (1978) Biochim.Biophys.Acta 523-494-505
5. De Metz, M., Vermeer, C., Soute, B.A.M. and Hemker, H.C. (1980) in *Vitamin K metabolism and vitamin K-dependent proteins*, (Suttie, J.W., ed.) pp.560-570, University Park Press, Baltimore
6. Stenflo, J. (1973) J.Biol.Chem. 248,6325-6332
7. Edman, P. and Henschen, A. (1975) in Protein Sequence Determination (Needleman, S.B., ed.), pp. 232-279, Springer Verlag, Berlin
8. Wiman, B. (1977) Eur.J.Biochem. 76,129-137
9. Hayashi, R., Moore, S. and Stein, W.H. (1973)J.Biol.Chem. 246,2296-2302
10. MacGillivray, R.T.A., Chung, D.W. and Davie, E.W. (1980) in Vitamin K metabolism and vitamin K-dependent proteins (Suttie, J.W.,ed.) 546-552, University Park Press, Baltimore

11. De Metz, M., Vermeer, C., Soute, B.A.M. van Scharrenburg, Slotboom, A.J. and Hemker, H.C. (1981) FEBS Lett. 123, 215-218
12. Rich, D.H., Lehrman, S.R., Kawai, M., Goodman, H.L. and Suttie, J.W. (1980) in Vitamin K metabolism and vitamin K-dependent protein (Suttie, J.W., ed.), pp. 471-479, University Park Press, Baltimore.

CHAPTER 4

PARTIAL PURIFICATION OF BOVINE LIVER VITAMIN K-DEPENDENT CARBOXYLASE BY IMMUNOSPECIFIC ADSORPTION ONTO ANTIFACTOR-X.

This work has been published,
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SUMMARY

The endogenous substrate for the vitamin K-dependent carboxylase, which accumulates in liver from warfarin-treated cows, consists mainly of precursors of prothrombin (± 20%) and factor X (± 70%). The carboxylase could be purified from the crude microsomal fraction a 100 fold by solubilization with Triton X-100 and immunospecific adsorption to antibodies against its endogenous substrate (precursors of factor X).

INTRODUCTION

Adminstration of vitamin K-antagonists induces the appearance of abnormal clotting factors in the blood (man, cow) or in the liver (rat). These abnormal clotting factors (descarboxyfactors) contain glutamic acid (Glu) residues instead of the γ-carboxyglutamic acid (Gla) residues, which occur in normal clotting factors (1). In human and bovine plasma 90% of the abnormal proteins consists of descarboxyprothrombin. Attempts to identify the rat liver abnormal proteins have been done in an in vitro vitamin K-dependent carboxylating enzyme system (carboxylase) and it was shown that only 25% of the carboxylated endogenous substrates bind to antiprothrombin antibodies (2).

Here, we describe the results of our investigations concerning the distribution of the various clotting factor precursors in preparations of vitamin K-dependent carboxylase from liver of warfarin-treated cows.

MATERIALS AND METHODS

Preparation of coagulation factors and antibodies. Bovine prothrombin was prepared and purified as in (3), factor IX as in (4) and factor X according to (5). The various coagulation factors were detected with the aid of one-stage coagulation assays (6-8). Antibodies against these proteins were raised in goats and extracted from the various sera by immunospecific adsorption to the respective Sepharose-bound antigens. The purified antibodies were eluted from the solid phase with 3 M NaCNS and gave single

precipitation lines against normal reference plasma in an Ouchterlony diffusion test (9). These antibodies wer coupled to CNBr-activated Sepharose (Pharmacia). Antiprothrombin-Sepharose slurry (1 ml) was able to neutralize the prothrombin content of 8 ml plasma. For antifactor IX--Sepharose and antifactor X-Sepharose these figures were 5 ml and 9 ml, respectively. The various antibody preparations did not bind detectable amounts of any of the other coagulation factors.

Preparation of carboxylase One year old cows were anticoagulated by the oral administration of warfarin (Sigma, 10 mg/kg daily) during 1 week. Cows were slaughtered and microsomes were prepared from the livers of normal and anticoagulated animals as in (10). The crude microsomes were suspended in a buffer containing 0.05 M KCl, 0.02 M Tris-HCl (pH 7.5) and 0.1% Triton X-100. After centrifugation at 100,000 x g for 1 h the pellet was solubilized by adding a buffer containing 1 M KCl, 0.02 M Tris-HCl(pH 7.5), 1 mM DTT, 0.5 mM EDTA and 0.5% Triton X-100. The final protein concentration was 15 mg/ml and insoluble material was removed by centrifugation (1 40 000 x g for 1.5 h). The supernatant of this run was designated as soluble carboxylase.

Measurement of carboxylase activity Unless indicated otherwise the vitamin K-dependent incorporation of $^{14}CO_2$ was measured by incubating soluble carboxylase (1 mg protein) and 20 µCi $NaH^{14}CO_3$ (Radiochemical Centre, Amersham) in reaction mixtures (0.25 ml) containing 0.15 M NaCl, 0.02 M Tris-HCl (pH 7.5), 1 mM DTT, 0.2 mM EDTA and 0.2% Triton X-100. The reaction was started by adding 15 µg chemically reduced vitamin K_1 (11) and the mixtures were incubated for 1 h at 25°C in sealed tubes. The reaction was stopped with 2 ml thrichloroacetic acid (10%) and the precipitates were washed and counted. The acid-soluble substrate Phe-Leu-Glu-Glu-Leu was prepared according to (12) and when it was present in the reaction mixtures the trichloroacetic acid supernatants were degassed at elevated temperatures before counting.

Gel electrophoresis and protein determination SDS-polyacrylamide gel electrophoresis was performed according to (13). Gels containing labeled products were sliced with a Gilson gel slicer and counted. Non-labeled gels

were stained with Coomassie blue. Protein concentrations were determined according to (14). The protein content of immobilized carboxylase was determined after elution of the solid phase with 6M urea in 2% SDS.

RESULTS AND DISCUSSION

In soluble rat carboxylase the incorporation of $^{14}CO_2$ in endogenous substrates is increased at least 20-fold when the rats are treated with warfarin 18 h before preparing the liver homogenates. We observed that in soluble carboxylase obtained from warfarin-treated cows the carboxylation of endogenous substrate was 16-fold higher (3000 dpm per mg of protein) than in carboxylase from normal cows (180 dpm/mg protein). These results suggested, that also in the cow liver some endogenous substrate (presumably clotting factor precursors) is accumulated during warfarin treatment and we

Table 1. Immunospecific adsorption of carboxylated reaction products.

Vitamin K-dependent incorporation of $^{14}CO_2$ was performed in standard reaction mixtures (1 ml). After incubation for 1 h at 25 °C, Sepharose-bound antibodies (0.4 ml slurry) were added to the various reaction mixtures and the tubes were rotated end-over-end overnight at 4 °C with 2% Triton X-100. The Sepharose beads were washed alternately 3 times with 1 M NaCl in 0.1 M acetate buffer (pH 4.0) and with 1 M NaCl in 0.1 M borate buffer (pH 8.0). Subsequently they were eluted with 6 M urea in 2% SDS, dialyzed against 0.1 M NaCl and counted. Non-adsorbed reaction mixtures (100%) contained 12,300 dpm. Doubling the amount of Sepharose-bound antibodies did not affect the amount of adsorbed proteins.

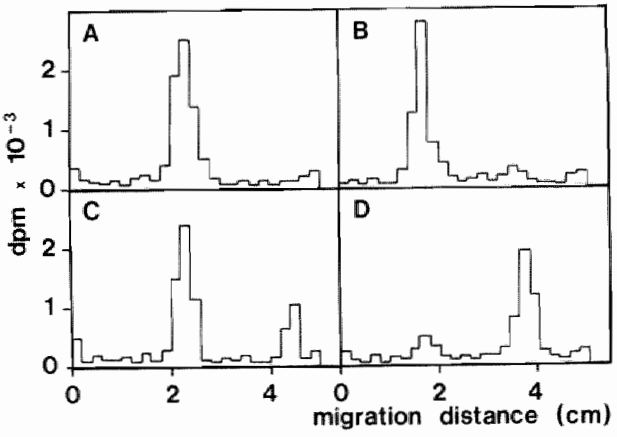
Sepharose-linked adsorbant	$^{14}CO_2$ incorporated into non-adsorbed proteins		$^{14}CO_2$ incorporated into adsorbed proteins	
	dpm	%	dpm	%
a) Antiprothrombin	9680	78.7	2595	21.2
b) Anti-factor X	3628	29.5	8511	69.2
c) Anti-factor IX	11414	92.8	959	7.8
d) a + b + c	1451	11.8	10860	88.3
e) Albumin	11795	95.9	37	0.3

therefore used carboxylase from anticoagulated cows for our studies concerning the nature of the bovine endogenous substrate.

Because of the presence of abundant proteolytic activity in bovine liver homogenates a direct determination of clotting factor precursors (e.g. with snake venoms) was not possible. In order to establish the nature of the endogenous substrate we therefore analyzed the ^{14}C-labeled products that were formed during the carboxylation reaction. The analysis was performed by adsorption to immobilized antibodies and, as is shown in table 1, the main part (69%) of the reaction product consists of factor X-like material, whereas antiprothrombin and antifactor IX bound 21 and 8% of the incorporated label, respectively. In a second experiment the factor X-like material was eluted from the antibodies and characterized further with the aid of SDS-polyacrylamide gel electrophoresis before and after reduction of the samples (fig.1). The non-reduced samples (A) showed a single band with M_r 55,000, which is in agreement with that of factor X (5,14). The app. M_r

Fig. 1. Sodiumdodecylsulphate-polyacrylamide gelelectrophoresis of carboxylated endogenous substrates.

Reaction mixtures (0.5 ml) were incubated for 1 h at 25 °C, mixed with 0.5 ml antifactor-X-Sepharose and rotated end-over-end at 4 °C overnight. The Sepharose was washed as described in the legend to table 1 and the labeled antigens were eluted with 3M NaCNS and dialyzed against 0.15 NaCl. The samples C and D were incubated at 37 °C for 1 h with snake venom (RVV_x) and SDS was added to all samples to 1% final concentration. (A) Non-reduced sample; (B) sample, reduced with 5% mercaptoethanol before electrophoresis; (C) non-reduced sample after treatment with RVV_x; (D) sample treated with RVV_x before reduction.



of the reduced protein (B) was found to be 65,000, which is higher than in the non-reduced gels and which shows that the factor X-like material consists of one single polypeptide chain. It turned out that E.carinatus venom, which is activating prothrombin, did not change the observed relative molecular masses (not shown). On the other hand the purified factor X-activating enzyme from Russell's viper venom (RVV_X) induced a small amount of low M_r material (15,000) in the non-reduced gel (C) whereas in the reduced sample (D) nearly all high M_r material had disappeared and was recovered at a position indicating an M_r of 25,000. We concluded that the factor X-like material is a factor X-precursor consisting of one polypeptide chain.

After cleavage of this chain by RVV_X, the molecule is held together by a disulfide bridge, which can be broken by reducing agents. Because the reaction mixture in which the carboxylation occurs is also slightly reducing, a small amount of low M_r material is observed in the non-reduced sample. The increase of the app.M_r in the reduced gels seems to be almost entirely due to changes in the light-chain material. A similar observation was made for the light chain of factor X (15,16). As plasma factor X contains two peptide chains, in vivo the carboxylation of the precursor probably precedes the conversion of one-chain factor X into two-chain factor X.

Taking into consideration that in bovine plasma circulating prothrombin and factor X are 1.4 and 0.28 μM, that their half-life times are 80 h and 30 h, respectively, and that factor X contains 2 more Gla residues than does prothrombin, it may be calculated that in vivo at least 1.5 more carboxylation reactions occur in prothrombin precursors than in factor X precursors. The experiments described above demonstrate that this situation is reversed in the in vitro carboxylating enzyme system. The relative abundance of factor X precursors in the carboxylase complex may be caused by a relatively high affinity of carboxylase for this substrate. During the preparation of the liver microsomes unbound factor X precursors might then displace other clotting factor precursors, present in the enzyme complex. A second possibility is that the prothrombin precursors are more susceptible to proteolytic degradation which may occur during the preparation of the microsomes. The available data provide no basis for distinguishing between these possibilities.

Since we knew that the endogenous substrate for the in vitro carboxylation reaction mainly consisted of precursors of factor X (69%) and prothrombin (21%), we started experiments in which we extracted the crude soluble carboxylase preparation with Sepharose-bound antibodies against factor X and prothrombin,respectively. Treatment with antiprothrombin removed 20% of the carboxylase activity, whereas antifactor X was able to bind as much as 62% of the total amount of carboxylase. A substantial amount of carboxylase activity was recovered on the Sepharose beads and because most of the microsomal proteins were removed by the washing procedure, the bound enzyme complex had been purified considerably. Solid phase carboxylase was assayed in the absence of Triton X-100 since detergents strongly inhibit its activity, even at low concentration.

A summary of the purification scheme is given in table 2.

Table 2. Purification of bovine vitamin K-dependent carboxylase.

Purification of bovine vitamin K-dependent carboxylase. The protein content of immobilized carboxylase was determined after elution of the solid phase with 6 M urea in 2% SDS. The carboxylation of endogenous substrate was measured in the various carboxylase preparations after 1 h incubation at 25 $^{\circ}$ C. When the carboxylase activity was measured with Phe-Leu-Glu-Glu-Leu the final purification was 115-fold.

Stage	Protein (mg)	Spec. activity (dpm/mg)	Recovery (%)	Purification (-fold)
Crude microsomes	2400	1420	100	1
Washed microsomes	1800	1885	110	1.5
Soluble carboxylase	1100	3006	97	2.1
Immobilized carboxylase	5	137276	20	97

REFERENCES

1. Stenflo, J., Fernlund, P., Egan, W. and Roepstorff, P. (1974) Proc.Natl.Acad.Sci.USA 71, 2730-2733
2. Willingham, A.K., Martin, S.L., Graves, C.B., Graham, G.G. and Munns, T.W. (1980) in: Vitamin K metabolsim and vitamin K-dependent proteins (Suttie, J.W. ed) pp.553-559, University Park Press, Baltimore.
3. Owen, W,G., Esmon, C.T. and Jackson, C.M. (1974) J.Biol.Chem. 249, 594-650
4. Fujikawa, K., Thompson, A.R., Legaz, M.E., Meyer, R.G. and Davie, E.W. (1973) Biochemistry 12, 4938-4945
5. Fujikawa, K., Legaz, M.E. and Davie, E.W. (1972) Biochemistry 11, 4882-4891
6. Vermeer, C., Soute, B.A.M. and Hemker, H.C. (1978) Thrombos.Res. 12, 713-716
7. Veltkamp, J.J., Drion, E.F. and Loeliger, E.A. (1968) Thromb. Diathes. Haemorrh. 19, 403-422
8. Vermeer, C., Soute, B.A.M. and Hemker, H.C. (1979) Thromb.Res. 14, 679-685
9. Ouchterlony, O, (1962) Progr.Allerg. 6, 30-154
10. Vermeer, C., Soute, B.A.M. and Hemker, H.C. (1978) Biochim.Biophys.Acta 523, 494-505
11. De Metz, M., Vermeer, C., Soute, B.A.M. and Hemker, H.C. (1980) in: Vitamin K metabolism and vitamin K-dependent protein (Suttie, J.W. ed) pp.560-570, University Park Press, Baltimore,
12. Suttie, J.W., Hageman, J.M., Lehrman, S.R. and Rick, D.H. (1976) J.Biol.Chem. 251, 5827-5830.
13. Laemmli, U.K. (1970) Nature 227, 680-685
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J.Biol.Chem. 193, 265-275
15. Jackson, C.M. (1972) Biochemistry 11, 4873-4881
16. Lindhout, M.J., Kop-Klaassen, B.H.M., Kop, J.M.M. and Hemker, H.C. (1978)Biochim.Biophys.Acta 533, 302-317

CHAPTER 5

PROPERTIES AND CHARACTERIZATION OF SOLID PHASE CARBOXYLASE

SUMMARY

With Sp-carboxylase the carboxylation of Phe-Leu-Glu-Glu-Leu proceeds with a linear rate for at least 3 h. The properties of the carboxylation reaction with Sp-carboxylase are similar to those found with solubilized microsomes from cow and rat liver. Sp-carboxylase contains several different proteins, as analyzed with SDS polyacrylamide gelelectorphoresis. Two proteins (M_r 45,000 and M_r 30,000) have vitamin K binding capacity. The carboxylase can be partly eluted from the solid phase, but further purification has not been achieved.

INTRODUCTION

In chapter 4 a 100 fold purification of vitamin K-dependent carboxylase from the microsomal fraction has been described. The purified enzyme is still attached to Sepharose beads and is therefore called: solid phase carboxylase (Sp-carboxylase). In this chapter some properties of the SP-carboxylase will be presented.

MATERIALS AND METHODS

Materials. Vitamin K, was obtained from Hoffman-LaRoche (Switzerland). ^{3}H labeled vitamin K was a kind gift from Hoffmann-LaRoche. 2-chloro-3-phytyl-naphtoquinone (chloro-K) was a kind gift of dr. J.W. Suttie. Triton X-100, p-hydroxymercuribenzoate, dithiothreitol, warfarin, vitamin E, superoxide dismutase and horse radish peroxidase were obtained from Sigma (USA). $NaH^{14}CO_3$ (40 Ci/mol) and Aquasol-2 were purchased from New England Nuclear (UK), the synthetic peptide Phe-Leu-Glu-Glu-Leu from Vega Fox (USA) and pyridoxalphosphate and catalase from Boehringer (FRG). DEAE-Sephadex was obtained from Pharmacia (Sweden) and AcA 22 from LKB (France). All other chemicals were from Merck (FRG). Vitamin K epoxide and vitamin K hydroquinone were prepared as described earlier (1).
Buffer A: 0.15 M NaCl, 20 mM Tris-HCl pH 7,5, 0.5 mM EDTA.

Preparation of carboxylase. Solid phase carboxylase (Sp-carboxylase) was prepared from the liver of warfarin-treated cows as described in chapter 4. The enzyme could be resolubilized from the Sepharose beads by having the carboxylation reaction proceeded in buffer A with 2 mM dithiothreitol, 0.2 mM vitamin K hydroquinone, 4 mM Phe-Leu-Glu-Glu-Leu and 5 mM $NaHCO_3$ for 5 h at 25°C. After incubation 1 M NaCl was added and the eluted carboxylase was dialyzed against buffer A before use.

Measurement of carboxylase activity. The vitamin K-dependent incorporation of $^{14}CO_2$ was measured by incubating reaction mixtures (0.25 ml) in buffer A containing 0.1 ml Sp-carboxylase (40 µg protein) or 10 µg resolubilized carboxylase, 20 µCi $NaH^{14}CO_3$, 2 mM dithiothreitol, and 0.2 mM vitamin K hydroquinone at 25°C. When the carboxylation of exogenous substrate was measured, Sp-carboxylase was preincubated in the presence of non labeled $NaHCO_3$ for 90 min, washed with buffer A and then assayed as described above. The reaction was stopped with 2 ml icecold trichloroacetic acid (10%) and the precipitates were washed and counted in Aquasol-2 in a Packard Tricarb scintillation counter. The trichloroacetic acid supernatants were degassed at elevated temperatures before counting. When the t-butylhydroperoxide-dependent carboxylation was assayed, the incorporation of $^{14}CO_2$ into endogenous and exogenous substrate was measured together.

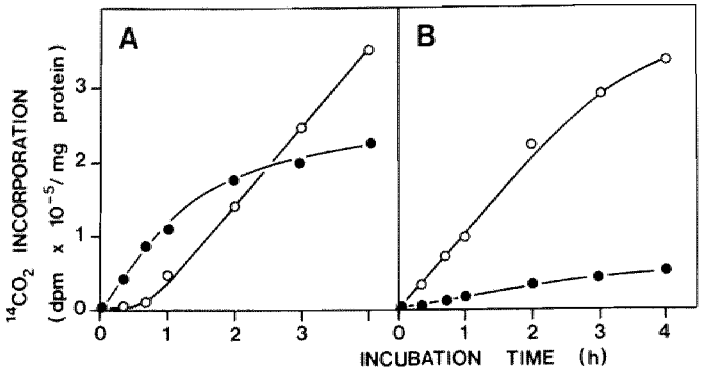
Protein determination and SDS polyacrylamide gelelectrophoresis. Protein concentrations were determined according to Lowry et al (2). SDS polyacrylamide gelelectrophoresis was performed according to Laemmli (3). Gels containing labeled products were sliced with a Gilson gel slicer and counted. Non labeled gels were stained with Coomassie brilliant blue.

RESULTS

Properties of Sp-carboxylase. When vitamin K hydroquinone and $H^{14}CO_3^-$ are added to Sp-carboxylase, the carboxylation of the endogenous substrate will start without delay (Fig. 1a). The time-course of this carboxylation reaction was not affected by adding 2 mM of the exogenous substrate Phe--Leu-Glu-Glu-Leu (data without peptide not shown), and the reaction rate

was constant for about 30 min. The incorporation of $^{14}CO_2$ into the exogenous substrate lagged behind for about 30 min, i.e. until about one-half of the endogenous substrate had been carboxylated. After the lag-phase, all carboxylase was still firmly bound to the Sepharose-beads but nevertheless, the carboxylation of exogenous substrate started and proceeded with a constant rate for more than 2 h (Fig. 1a).

Fig. 1. The carboxylation of endogenous (●——●) and exogenous (○——○) substrate by Sp-carboxylase.



A, Sp-carboxylase was incubated in the presence of vitamin K hydroquinone, $H^{14}CO_3^-$ and pentapeptide and the incorporation of $^{14}CO_2$ into the various substrates was measured. B, Sp-carboxylase was preincubated at 25 °C in the presence of vitamin K hydroquinone and nonlabeled HCO_3^- for 90 min, washed with 0.15 M NaCl, and incubated with vitamin K hydroquinone, $H^{14}CO_3^-$ and peptide substrate as described above.

Because we also wanted to assay the carboxylation in a system that was independent of the variable conditions induced by the preferential carboxylation of the endogenous substrate, we preincubated Sp-carboxylase in the presence of vitamin K hydroquinone and nonlabeled $NaHCO_3$ and in the absence of the peptide substrate for 90 min at 25°C. After this period the Sepharose beads were washed and could be used for the carboxylation of the pentapeptide. Now the reaction showed no lag phase and proceeded in a linear way for more than 3 h (Fig. 1b). It turned out that during the preincubation no detectable amount of enzyme was lost. The preincubated Sp-carboxylase was used in all experiments mentioned below, when the vitamin K-dependent carboxylation was measured.

Well known inhibitors and stimulators of rat liver carboxylase (4) were tested with Sp-carboxylase (Table I). The results are similar to those found with rat carboxylase and bovine carboxylase.

Table I. Comparision between the vitamin K-dependent and the t-butylhydroperoxide-dependent carboxylation.

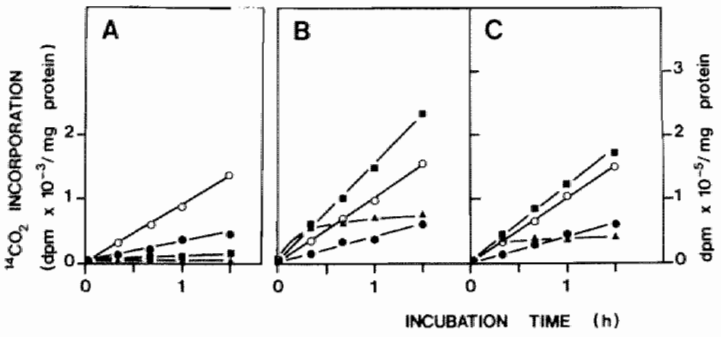
Addition to standard reaction mixture	Vitamin K-dependent carboxylation (% of control)	t-butylhydroperoxide-dependent carboxylation (% of control)
none	100	100
none, dithiotreitol omitted	26	3
chloro-K (0.1 mM)	30	8
warfarin (1 mM)	62	20
p-hydroxymercuribenzoate (1 mM)	4	0
pyridoxalphosphate (2 mM)	138	114
EDTA (2 mM)	102	104

A number of chemicals, known to either inhibit or stimulate the vitamin K-dependent carboxylation was assayed for their effect on the t-butylhydroperoxide-driven carboxylation. In the latter case vitamin k hydroquinone was replaced by 2.5 mM t-butylhydroperoxide. The control values (100%) were: 2300 dpm per mg protein per min for the vitamin K-dependent reaction and 420 dpm per mg protein per min for the t-butylhydroperoxide-dependent reaction.

When we compared Sp-carboxylase with the vitamin K-dependent carboxylase present in solubilized microsomes, we observed three differences a) Sp-carboxylase is far more active with reduced vitamin K than with non reduced vitamin K. The activity of Sp-carboxylase in the presence of vitamin K or vitamin K epoxide is respectively 20% and 5% of the activity observed in the presence of vitamin K hydroquinone. b) The K_m of the synthetic pentapeptide is 11 mM in the microsomal extract and 3 mM in Sp-carboxylase. The K_m of "CO_2" is 0.2 mM in the microsomal extract and 0.8 mM in Sp-carboxylase. c) Sp-carboxylase is more stable at elevated temperatures than carboxylase in the microsomal extract (Fig. 2).

The highest activity was observed at 35°C and at this temperature the reaction rate was constant for more than 2 hours. When carboxylase was eluted from the Sepharose beads (see Materials and Methods) the enzyme remained fully active at 35°C, whereas the non-purified carboxylase in solubilized microsomes is rapidly destroyed at this temperature.

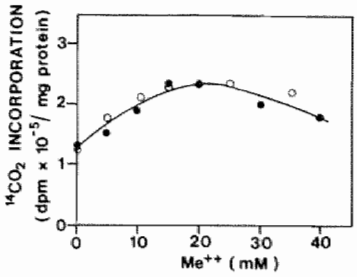
Fig. 2. Activity of various carboxylase preparations during time course studies at 15 °C (●——●), 25 °C (○——○), 35° C (■——■) and 45 ° C (▲——▲).



A, carboxylase in solubilized microsomes; B, Sp-carboxylase; C, resolubilized carboxylase. The incubations conditions were as described in Materials and Methods.

Effect of divalent metal ions. Divalent metalions had various effects on the carboxylation reaction. Most of them were found to be inhibitory when added at concentrations higher than 0.1 mM (Cd^{++}, Zn^{++}, Cu^{++}, Sn^{++}). Half maximal inhibition with Fe^{++} was observed at a concentration of 10 μM. The inhibition by Fe^{2+} was non competitive with the reaction components (Phe--Leu-Glu-Glu-Leu, Vitamin K hydroquinone, $NaHCO_3$ and O_2). Mg^{++} and Ba^{++} did not affect the carboxylation reaction at concentrations below 50 mM, whereas Ca^{++} and Mn^{++} were stimulating (Fig. 3). Ca^{++} and Mn^{++} ions did not affect the K_m of Phe-Leu-Glu-Glu-Leu, which is present in a suboptimal concentration in the reaction mixture. Our efforts to prepare a metal ion-dependent carboxylase with chelating agents were not succesfull (not shown).

Fig. 3. The stimulation of the vitamin K-dependent carboxylation by Ca^{++} and Mn^{++} ions.



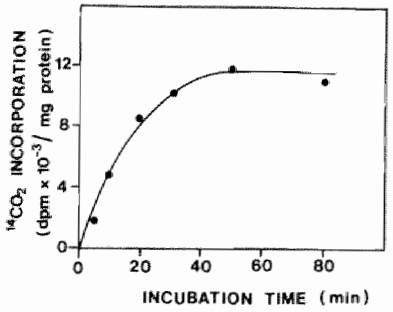
The Carboxylation of Phe-Leu-Glu-Glu-Leu was measured with Sp-carboxylase for 1 h at 25 °C in the presence of Ca^{++}(O——O) and Mn^{++} (●——●) as described in Materials and Methods.

Effect of various scavengers and the possible involvement of a hydroperoxide intermediate in the carboxylation reaction. Radical scavengers are known inhibitors of carboxylase in the rat system. We also found inhibition by various anitoxidantia (phenol, naphtol, ethanol, vitamin E). Scavengers, which are more or less specific for .OH (mannitol, glucose, tryptophan), singlet O_2 (histidine, azide), $.O_2^-$ (superoxide dismutase) and H_2O_2 (catalase), were not found to affect the carboxylation reacton at concentrations known to be effective in other systems (5,6). The inhibition by peroxidases (7) could be confirmed with Sp-carboxylase. Furthermore t-butylhydroperoxide could replace vitamin K hydroquinone in the reaction mixture, as has been reported for the rat system (8). This t-butylhydroperoxide dependent reaction was stimulated or inhibited by the same reagents as the vitamin K-dependent reaction (table I). A time course of the t-butylhydroperoxide-dependent carboxylation is given in fig. 4.

The reaction velocity decreased after about 30 min, but a second addition of t-butylhydroperoxide again stimulated the carboxylation reaction, indicating that the peroxide concentration had decreased during the first incubation. The total carboxylation after the renewed addition was less, however, (5000 dpm/mg) than after the first addition (12.000 dpm/mg). this may be due to inactivation of Sp-carboxylase by

t-butylhydroperoxide. It could be demonstrated that Sp-carboxylase, incubated with 20 mM t-butylhydroperoxide for 60 min and subsequently washed with buffer A, was completely inactive (as measured with the vitamin K--dependent and t-butylhydroperoxide-dependent incorporation of CO_2).

Fig. 4. Time course of the t-butylhydroperoxide-dependent carboxylation.

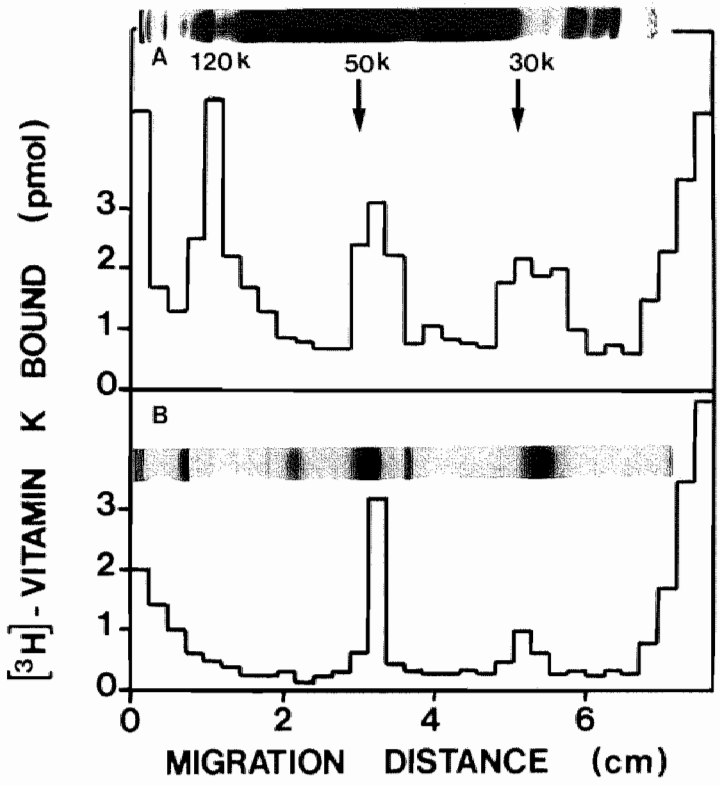


The carboxylation was measured with Sp-carboxylase as described in Materials and Methods. Vitamin K hydroquinone was replaced by 2.5 mM t-butylhydroperoxide.

Characterization of Sp-carboxylase. The protein part of Sp-carboxylase was eluted from the Sepahrose with 6 M urea and 2% SDS and analyzed with the aid of SDS polycrylamide gel-electrophoresis. On these gels we observed several protein bands (fig. 5). Two major bands (M_r 50,000 and M_r 30,000) are not part of the carboxylase complex, but are impurities that elute from the antibodies linked to the Sepahrose (even when no carboxylase is bound to it). Up till now we do not know which of the other proteins belong to the carboxylase complex. It seem plausible, however, that at least two different proteins are part of the complex, as two proteins (M_r 45, 000 and 30,000) bound ^3H labeled vitamin K (Fig. 5). The vitamin K binding capacity of the proteins in Sp-carboxylase which was analyzed on SDS gels and which is expressed as dpm per mg protein, was at least 50 times higher than was the binding capacity of the proteins in solubilized microsomes.

Elution of the enzyme from the solid phase. The enzyme is linked to the Sepharose via a strong antigen-antibody interaction. The antibody is covalently attached to the Sepahrose and the antigen (endogenous substrate)

Fig. 5. The binding of ^{3}H labeled vitamin K_1 on microsomal proteins (A) and on Sp-carboxylase (B) as analyzed by SDS-polyacrylamide gelelectrophoresis.



Solubilized microsomes (0.5 mg of protein) and Sp-carboxylase, eluted from the solid phase with 6 M urea and 2 % SDS (0.05 mg of protein), were analyzed on SDS gels as desribed in Materials and Methods.When the gels had to be sliced for counting (see Materials and Methods) the procedure was as follows. Solubilized microsomes (0.5 mg of protein) and Sp-carboxylase (0.01 mg of protein) were incubated with 20 µCi ^{3}H labeled vitamin K_1 (300 Ci/mol) for 2 h at 25 °C and dialyzed overnight against 0.1 % SDS, 0.1 M glycine and 0.05 M Tris-HCl pH 8.0 before electrophoresis.

is tightly complexed to the enzyme. Conventional methods for the breakage of the antigen-antibody interaction were not succesful in the resolubilization of carboxylase, because the enzyme activity was irreversibly lost. By having the carboxylation reaction proceed in the presence of vitamin K hydroquinone and Phe-Leu-Glu-Glu-Leu (see Materials and Methods) up to 20% of the carboxylase activity could be regained in solution. In the presence of an excess of antigen (factor X) the eluted activity raised to 30%. The factor X could be removed from the carboxylase with the aid of DEAE Sephadex. The carboxylase preparation thus obtained had a high molecular weight (M_r 700,000), as estimated with column chromatography (AcA 22) and did not contain the major impurities (M_r 50,000 and M_r 30,000) present in Sp-carboxylase. A further purification seems impossible without breaking the high molecular weight aggregate. Until now this could not be achieved without a substantial loss of enzyme activity.

DISCUSSION

Sp-carboxylase is strongly attached to the Sepharose beads via its endogenous substrate (a factor X precursor). The endogenous substrate can still be carboxylated and it seems plausible that the substrate, is released from the carboxylating enzyme after being carboxylated. Indeed we observed that carboxylase was partly eluted from the solid phase by having the carboxylation reaction proceeded. It is not known whether all 13 carboxylatable glutamic acid residues in the factor X precursor are carboxylated. However, because the main part of carboxylase remains attached to the solid phase after carboxylation of the endogenous substrate, we think that the substrate is carboxylated incompletely.

A further purification could not be achieved with the carboxylase eluted from the solid phase, as this carboxylase preparation is a high molecular weight structure, which could not be cleaved without a loss of enzyme activity.

The vitamin K-dependent carboxylation reaction can be studied independent of the variable conditions induced by the presence of endogenous substrate by using preincubated Sp-carboxylase. The carboxylation

rate of Phe-Leu-Glu-Glu-Leu with the preincubated Sp-carboxylase is linear for at least 3 h and the properties of this reaction are similar to those of a non-purified microsomal preparation. Non reduced forms of vitamin K however, are less active than vitamin K hydroquinone, indicating that Sp-carboxylase has a low amount of vitamin K reductase. Most features of the vitamin K-dependent carboxylation reaction known from the rat system (such as the possible involvement of a hydroperoxide intermediate and the stimulation by some divalent metal ions), could also be demonstrated with bovine Sp-carboxylase. The t-butylhydroperoxide-dependent carboxylation could also be demonstrated with Sp-carboxylase and the requirements for this type of carboxylation were similar to those for the vitamin K-dependent carboxylation. This finding supports the idea that a hydroperoxide intermediate of vitamin K is driving the carboxylation reaction (7). As has been stated already by Larson and Suttie (9) the stimulation by metal ions is probably due to an unknown activation of the in vitro system and not to a specific metal ion requirement of the enzyme. The strong inhibition by Fe^{2+} ions can readily be explained by assuming that Fe^{2+} acts as a peroxidase in our system. Evidence for this hypothesis will be presented in chapter 8.

When the protein part of Sp-carboxylase was analyzed with SDS polyacrylamide gelelectrophoresis several protein bands were observed. Two proteins were binding ^{3}H labeled vitamin K (M_r 45 000 and M_r 30 000). It is not known if these are the only proteins required for the proper function of carboxylase. A further characterization of carboxylase is hampered by the fact that a further purification of the active enzyme cannot be achieved at this moment.

REFERENCES

De Metz, M., Vermeer, C., Soute, B.A.M. and Hemker, H.C. (1980) in: Vitamin K metabolism and vitamin K dependent proteins (Suttie, L.W. ed.), University Park Press, Baltimore, 560-570

2. Lowry, O.H., Rosebrough, N.J., Favor, A.L. and Randall, R.J. (1951) J.Biol.Chem. 193, 265-275
3. Laemmli, V.K. (1970), Nature 227, 680-685
4. Suttie,J.W. (1980) C.R.C. Crit.Rev.Biochem. 8, 191-223
5. Kellogg, E.W. and Fridovich, I. (1977) J.Biol.Chem. 252, 6721-6728
6. McPhail, L.C., Ambrusso, D.R., Pabst, H.J. and Johnston, R.B. (1979) in: Biochemical and Clinical Aspects of Oxygen (Laughey, W.S. ed.) pp. 709-723. Academic Press, New York.
7. Larson, A.E. and suttie, J.W. (1978) Proc.Natl.Acad.Sci.USA 75, 5413--5416
8. Suttie, J.W., Larson, A.E., Canfield, L.M. and Carlisle, T.L. (1978) Fed.Proc. 37, 2605-2609
9. Larson, A.E. and Suttie, J.W. (1980) FEBS Lett. 118, 95-98

CHAPTER 6

IDENTIFICATION OF PHOSPHOLIPID AS AN ESSENTIAL PART OF BOVINE VITAMIN K-DEPENDENT CARBOXYLASE.

This work has been published,
De Metz, M., Vermeer, C., Soute, B.A.M. and Hemker, H.C. (1981) J.Biol. Chem. 256, 10843-10846

SUMMARY

Vitamin K-dependent carboxylase from bovine liver contains phospholipid (primarily phosphatidylcholine), which is essential for its in vitro activity. Sepharose-bound carboxylase can be depleted of phospholipids, either by washing the enzyme with detergents or by phospholipase treatment. The enzyme can be reconstituted by adding mixed micelles of phosphatidylcholine and cholate to the Sepahrose-bound proteins.

INTRODUCTION

Vitamin K is required for the post-translational carboxylation of a number of glutamic acid residues in many proteins. Several reviews about this reaction have been published recently (1-3). The carboxylating enzyme system (carboxylase) is obtained from the rough microsomal fraction of hepatic cells (4) and can be extracted therefrom with detergents or bile salts (5). Until now, the purification of carboxylase from this extract has not been accomplished, mainly because the enzyme activity is lost during the purification procedure (5,6). It has been suggested therefore, that the enzyme is constituted of at least two components, both of which are required for the carboxylation reaction (7).

We have developed a soluble carboxylating enzyme system from the liver of warfarin-treated cows. This bovine carboxylase was purified more than 100-fold by immunospecific adsorption to antibodies against its endogenous substrate (chapter 4, 8). In this chapter we present evidence that phospholipids are an essential part of this partly purified carboxylase.

MATERIALS AND METHODS

Materials. Buffer A: 0.15 M NaCl, 20 mM Tris-HCl, pH 7.5. Triton X-100, cholate, dithiothreitol and bovine albumin (essentially fatty acid free), PI* and SPH were obtained from Sigma and vitamin K_1 from Hoffmann-La

Roche. $NaH^{14}CO_3$ (40 mCi/mmol) and Aquasol-2 were purchased from New England Nuclear and the synthetic pentapeptide Phe-Leu-Glu-Glu-Leu from Vega Fox. Phospholipase A_2 (from Naja naja), phospholipase C (from Bacillus cereus) and phospholipase D (from Savoy cabbage) were prepared as described by Zwaal et al. (9). PC was prepared from egg yolk (10) and used for the preparation of phosphatidic acid, PS, PE and PG (11). Diglycerides were prepared from PC by hydrolysis with phospholipase C and lyso-PC and free fatty acids by hydrolysis with phospholipase A_2. The phospholipids were added to the various phospholipases in the form of mixed micelles, PC and cholate being present in a 1:1 ratio (w/w). The micelles were prepared by evaporating the solvent from the various (phospho)lipids and resuspending the residue in buffer A containing Triton X-100 or cholate as indicated. The suspension was sonicated subsequently until a clear solution was obtained. During the phospholipase treatment the reaction conditions were as described below. The hydrolysis products were separated with the aid of silica thin layer chromatography, using a mobile phase containing chloroform: methanol: NH_4OH: water in a ratio of 90 : 54 : 5.5 : 5.5 (v/v). The lipids were extracted from the silica with a mixture containing equal volumes of chloroform and ethanol.

Preparation of carboxylase. Carboxylase was prepared and solubilized from the microsomal fraction of the livers of warfarin-treated cows and adsorbed from the solution with antifactor X-Sepharose (chapter 4). This solid-phase carboxylase (Sp-carboxylase) was used for most of the experiments, and contained 150 µg of protein per ml Sepharose slurry.

Measurement of carboxylase activity. The vitamin K-dependent incorporation of $^{14}CO_2$ was measured by incubating reaction mixtures (0.25 ml) containing either 0.1 ml of Sp-carboxylase or 10 µg of re-solubilized carboxylase and 2 mM dithriothreitol, 0.2 mM vitamin K hydroquinone, 20 µCi $NaH^{14}CO_3$ and buffer A at 25°C. When the carboxylation of exogenous substrate was measured, Sp-carboxylase was preincubated in the presence of non-labeled $NaHCO_3$ (instead of $NaH^{14}CO_3$) for 90 min, washed with buffer A and then assayed as described above. The reaction was stopped with 2 ml icecold trichloroacetic acid (10 % w/w) and the precipitates were washed and counted in Aquasol-2 in a Packard Tri-Carb scintillation counter. The

trichloroacetic acid supernatants were degassed at elevated temperatures before counting

Treatment with detergents and phospholipases. For the treatment of Sp-carboxylase with detergents, 0.5 ml of the Sepahrose-bound enzyme was incubated for 30 min at 25°C in 1.5 ml of buffer A and detergents as indicated. The Sepharose beads were washed subsequently with 20 ml of buffer A containing 2 mM EDTA and 2 mg/ml of bovine albumin and once more with 20 ml of buffer A containing 0.5 mM EDTA. Incubations with phospholipase A_2 and phospholipase C were carried out in buffer A containing 10 mM $CaCl_2$. Incubations with phospholipase D were carried out in 50 mM sodiumacetate, 30 mM NaCl, 50 mM $CaCl_2$, pH 6.3. Sp-carboxylase (0.5 ml) was supplemented with 1.5 ml of buffer A containing 5 I.U. of phospholipase and incubated for 1 h at 37°C. After the incubation the solid-phase enzyme was washed in a manner similar to that used after the treatment with detergents.

Phospholipid and protein determination. Phospholipids were extracted from carboxylase according to the method of Bligh and Dyer (12), separated by two-dimensional thin layer chromatography (13), and quantitated by determining the amount of phosphate present in each spot (14). Protein concentrations were determined according to Lowry et al. (15). The protein content of Sp-carboxylase was measured after eluting the Sepharose beads with 6 M urea in 2% sodium dodecyl sulphate.

RESULTS

Phospholipid content of Sp-carboxylase. Sp-carboxylase is prepared by adsorbing solubilized microsomes onto antifactor X-Sepharose. The factor X-precursors present in the soluble preparation (chapter 4, 8) bind to the antibodies, and because most of the carboxylase is tightly complexed to these factor X-precursors, the enzyme-substrate complex is bound to the Sepharose beads. The enzyme preparation thus obtained contained 72% protein and 28% phospholipid. With the aid of two-dimensional thin layer chromatography, more than 95% of these phospholipids could be identified as PC.

Effect of detergents of Sp-carboxylase. The surfactants Triton X-100, cholate and deoxycholate, which are commonly used for the extraction of carboxylase from the microsomal membranes, strongly inhibited Sp-carboxylase. This inhibition could be prevented by adding a crude extract of microsomal phospholipids to the insolubilized enzyme. Since the bulk of detergent is easily separated from Sp-carboxylase by washing the Sepharose beads, we were able to demonstrate the reversibility of the detergent-induced inactivation (Table 1).

At low detergent concentrations, a substantial loss of enzyme activity was observed, but after washing the Sepahrose beads with a buffer containing 1 mM EDTA, much of the carboxylating activity was restored and

Table 1. The effect of detergents on the carboxylation rate of Phe-Leu-Glu-Glu-Leu.

One ml of Sp-carboxylase was incubated with detergent (as indicated) for 10 min at 30 °C. The Sepharose beads were washed as described under Materials and Methods, and the carboxylase activity was measured and expressed as a percentage of the control value (115,000 dpm per mg of protein). The concentration of exogenous phospholipids was 1 mg/ml, and they were added to the enzyme as mixed micelles of phospholipids and cholate. In these micelles the ratio of phospholipid:cholate was 1:1 (w/w). The incorporation of $^{14}CO_2$ was assessed after 1 h at 25 °C.

Before washing		After washing		
Detergent added	Activity	Phospholipid/protein ratio (w/w)	Carboxylase activity	
			Minus phospholipids	Plus phospholipids
	% of control		% of control	
None	100	0.40	105	115
Cholate (0.1%)	50	0.38	95	120
Cholate (0.5%)	3	0.06	12	65
Deoxycholate (0.1%)	12	0.25	62	84
Deoxycholate (0.5%)	2	0.04	10	41
Triton X-100 (0.02%)	30	0.30	70	90
Triton X-100 (0.1%)	1	0.02	16	42

no significant loss of phospholipids could be measured. At higher detergent concentrations, however, nearly all carboxylase activity was lost. After the washing procedure about 90% of the phospholipids had been removed and only 10-15% of the enzyme activity was left. After recombining the washed Sepharose beads with microsomal phospholipids about one-half of the original carboxylase activity could be regained.

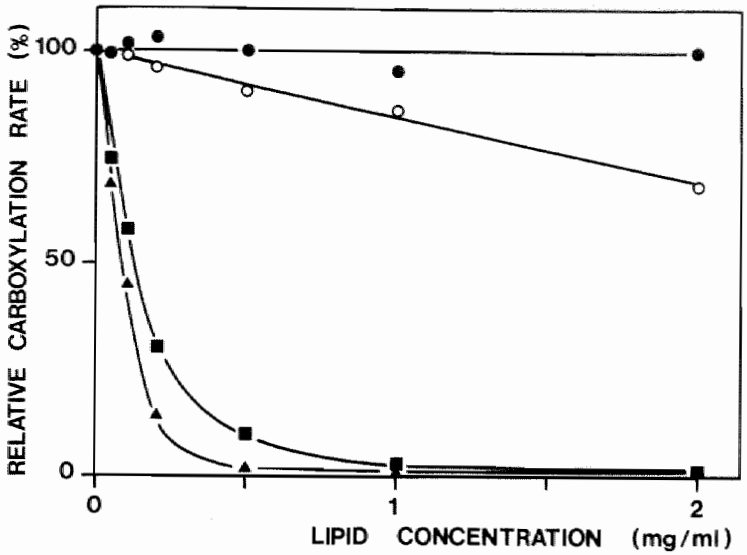
The effect of phospholipases on Sp-carboxylase. Sp-carboxylase was incubated in the presence of a number of phospholipases, which were subsequently removed by washing the solid-phase enzyme as described in Materials and Methods. In all cases the carboxylating activity was essentially destroyed (Table 2), which indicates that either carboxylase requires intact phospholipids or the degradation products of the endogenous phospholipids (almost exclusively PC) act as inhibitors of carboxylase.

Table 2. The effect of phospholipases on the carboxylation rate of Phe-Leu-Glu-Glu-Leu.

One ml of Sp-carboxylase was incubated with 1 mg of phospholipase for 1 h at 30°C. The Sepharose beads were washed as described under "Materials and Methods", and the carboxylase activity was measured and expressed as a percentage of the control value (115,000 dpm per mg protein). The concentration of exogenous phospholipids was 1 mg/ml, and they were added to the enzyme as mixed micelles of phospholipids and cholate as described in the legend to Table I. The incorporation of $^{14}CO_2$ was assessed after 1 h at 25 °C.

Before washing		After washing		
Phospholipase used	Carboxylase activity	Phospholipid/protein ratio (w/w)	Carboxylase activity	
			Minus phospholipids	Plus phospholipids
	% of control		% of control	
Phospholipase A_2	0	0.01	4	40
Phospholipase C	1	0.06	6	66
Phospholipase D	0	0.29	1	22

Fig. 1. Inhibition of Sp-carboxylase by phospholipid degradation products.



Sp-carboxylase was supplemented with mixed micelles of cholate and either lyso-PC (●——●), diglycerides (○——○), phosphatidic acid (■——■), or free fatty acids (▲——▲) in a 1:1 ratio (w/w). Subsequently, the carboxylase activity was measured in the presence of 1 mM EDTA. The incorporation of $^{14}CO_2$ was assessed after 1 h at 25 °C.

The influence of the various split products of PC was therefore assessed. It appeared that the water-soluble choline and cholinephosphate (which are formed by phospholipase D and C, respectively) are readily washed out from the Sepahrose beads. Free fatty acids and phosphatidic acid (formed by phospholipase A_2 and D respectively) are strong inhibitors of carboxylase (Fig. 1), whereas lyso-PC and diglycerides (resulting from the action of phospholipase A_2 and C respectively) hardly influence the vitamin K-dependent carboxylation. Moreover, when using a washing buffer containing bovine albumin and EDTA, the free fatty acids (as well as lyso-PC) were efficiently removed from the solid-phase enzyme. No washing procedure could be developed, however, by which any carboxylase activity was recovered. On the other hand, in all cases the enzyme activity could be partially restored by adding crude microsomal phospholipids. The recovery was about 50% except in the case of phospholipase D, where one of the split products

(phosphatidic acid) could not be completely washed out. Since phosphatidic acid is a strong inhibitor of carboxylase (Fig. 1), this low recovery of carboxylase activity is to be expected in this case.

Reconsitution of phospholipid-depleted Sp-carboxylase with purified phospholipids. Sp-carboxylase was depleted from its endogenous phospholipids by incubation with phospholipase C and washed as described above. A number of purified phospholipids was assayed subsequently for their ability to reconstitute the active enzyme (Table 3).

Table 3. The dependance of Sp-carboxylase on purified phospholipids.

Phospholipid-depleted Sp-carboxylase was prepared with the aid of phospholipase C as described in the legend to Table II. The various phospholipids were added to the enzyme as mixed micelles with cholate in a 1:1 ratio (w/w). The final phospholipid concentration was 1 mg/ml. The results are expressed as a percentage of the control value (115,000 dpm per mg of protein). The incorporation of $^{14}CO_2$ was assessed after 1 h at 25 °C.

Phospholipid added	Carboxylase activity in	
	Sp-carboxylase	Phospholipid-depleted Sp-carboxylase
	% of control	
None	100	5
Crude microsomal extract	104	70
Phosphatidylcholine	110	84
Lyso-phosphatidylcholine	98	77
Sphingomyelin	90	50
Phosphatidylethanolamine	50	19
Phosphatidylinositol	3	0
Phosphatidylserine	2	0
Phosphatidylglycerol	2	0
Phosphatidic acid	4	0

The neutral phospholipids SPH, PC, PE and SPH, as well as lyso-PC (the splitproduct that is formed when PC is digested with phospholipase A_2) were able to partially restore the activity of phospholipid-depleted carboxylase. A maximal effect was obtained when the phospholipids were added in a

concentration of 0.5 - 2 mg/ml as mixed micelles with cholate. PC was the phospholipid most effective in restoring the enzyme activity (up to 84%), whereas mixtures of PC and other phospholipids induced a less marked stimulation (data not shown). Negatively charged phospholipids such as PI or PG as well as phosphatidic acid did not restore any carboxylating activity. On the other hand, when added to the phospholipid-containing native enzyme, these negatively charged phospholipids strongly inhibited carboxylase. Most probably this inhibition is caused by a rapid exchange between the phospholipids in carboxylase and those in the added micelles. The presence of cholate does not seem to be an absolute requirement for the enzyme, since the removal of cholate by dialysis had no effect on the activity of the reconstituted enzyme preparation.

DISCUSSION

Sp-carboxylase is more than 100-fold purified as compared to a microsomal suspension and its properties are not very different from the latter (chapter 5). It was , however, inhibited by low concentrations of non-ionic detergents.

Analysis of the Sepharose-bound enzyme showed that it contains about 30% (w/w) phospholipids (almost exclusively PC). When we removed the phospholipid moiety, either by washing carboxylase with detergents or by destroying the phospholipids with various phospholipases, the carboxylase activity had disappeared. It could be restored by addding certain exogenous phospholipids in the form of mixed micelles, with cholate present in 1:1 ratio (v/v). Vesicles of phospholipids alone were not effective in this respect, probably because in the absence of detergent there is no transfer of phospholipids from the vesicles to the Sepharose-bound proteins. PC turned out to be the most effective in restoring carboxylase activity. This is in agreement with our observation that the natural phospholipid present in Sp-carboxylase is PC. When analyzed by SDS gel electrophoresis, Sp--carboxylase contained several bands (chapter 5) and it is not known whether all these proteins are required for carboxylase activity. The proteins present at this stage of purification are tightly complexed, however, and we have not succeeded in isolating the various components

without a complete loss of activity. Whether all these proteins require phospholipid for their proper function cannot be conluded therefore.

When 1 mM EDTA was present in the buffers during the separation of the enzyme proteins and phospholipids and during their reconstitution, the recovery of active carboxylase could be greatly improved. As it is well known (16) that integral membrane proteins may aggregate upon removal of their phospholipids, the effect of EDTA might be a prevention of irreversible aggregation or denaturation of carboxylase when the latter is depleted of phospholipids.

The fact that phospholipids are an integral part of carboxylase may have implications for the purification of the enzyme. Up to now, several investigators (5,6) have tried to purify carboxylase, but after fractionating the enzyme in a number of ways they observed that it had lost its biological activity. One of the possible explanations for this phenomenon might be that during the fractionation procedures the proteins of the enzyme complex are separated from the phospholipid moiety. In order to exclude this pitfall, it seems advisable to add mixed micelles of PC and cholate after each purification step to the fraction when they are assayed for carboxylase activity.

* The abbreviations used are: PI, phosphatidylinositol; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SPH, sphingomyelin

REFERENCES

1. Stenflo, J., and Suttie, J.W. (1977) Ann.Rev.Biochem. 46, 157-172
2. Olson, R.E. and Suttie, J.W. (1978) Vitam.Horm. 35, 59-172
3. Suttie, J.W. (1980) C.R.C. Crit.Rev.Biochem. 8, 191-223
4. Helgeland, L. (1977) Biochim.Biophys.Acta 499, 181-193
5. Johnson, B.C. (1980) Methods Enzymol. 67, 165-180

6. Wallin, R., Canfield, L.M., Sinsky, T.A. and Suttie, J.W. (1980) in Vitamin K metabolsim and vitamin K-dependent proteins (Suttie, J.W. ed.) pp. 490-499, University Park Press, Baltimore
7. Price, J.A., Bartels, V.L., Chiu, A., Delaney, R., and Johnson, B.C. (1980) J.Biol.Chem. 255, 1808-1811
8. De Metz, M., Vermeer, C., Soute, B.A.M., van Scharrenburg, G.J.M., Slotboom, A.J. and Hemker, H.C. (1981) FEBS Letters 123, 215-218
9. Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and van Deenen, L.L.M. (1975) Biochim.Biophys.Acta 406, 83-96
10. Singleton, W.S., Gray, M.S., Brown, N.L. and White, J.L. (1965) J.Am.Oil Chem.Soc. 42, 53-56
11. Comfurius, P. and Zwaal, R.F.A. (1977) Biochim.Biophys.Acta 488, 36-42
12. Bligh, E.G. and Dyer, W.J. (1959) Can.J.Biochem.Physiol. 39, 911-917
13. Broekhuijse, R.M. (1969) Clin.Chim.Acta 23, 457-461
14. Chen, P.S., Toribara, T.Y. and Warner, M. (1965) Anal.Chem.28, 1756-1758
15. Lowry, O.M., Rosebrough, N.J., Farr, A.L. and Randall, R.J.(1951) J.Biol.Chem. 193, 265-275
16. Helenius, A. and Simons, K. (1975) Biochim.Biophys.Acta 415, 29-79

CHAPTER 7

THE INHIBITION OF VITAMIN K-DEPENDENT CARBOXYLASE BY CYANIDE

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M. de Metz, B.A.M. Soute, H.C. Hemker and C. Vermeer, FEBS Lett.137, 253-256

SUMMARY

The vitamin K-dependent carboxylation and the t-butylhydroperoxide-dependent carboxylation are inhibited by cyanide, whereas the epoxidation of vitamin k hydroquinone is not inhibited. The inhibition was non-linear and competitive with CO_2. Haem could not be detected in Sp-carboxylase.

INTRODUCTION

The formation of γ-carboxyglutamic acid residues from glutamic acid residues is a vitamin K-dependent carboxylation reaction. The reaction has been demonstrated in various species and tissues (1,2), but the most extensive studies have been performed in the microsomal fraction of rat liver. It is generally assumed that the carboxylation reaction is coupled to the epoxidation of reduced vitamin K (3,4), but the reaction mechanism is still unclear. The purification of the carboxylating enzyme (carboxylase) from rat liver seems to be difficult and has not been reported until now. The possible involvement of other cofactors, such as haem groups (5-7) is therefore still a matter of dispute. The main argument for the involvement of haem in the carboxylation reaction was the observation that cyanide inhibits rat carboxylase (5,8). This inhibition has not been further analyzed, however, whereas other common haem ligands such as azide and carbon monoxide do not inhibit the vitamin K-dependent carboxylation (1).

We have developed a carboxylating enzyme system from the livers of warfarin-treated cows and obtained a 100-fold purification of the microsomal enzyme by immunospecific adsorption onto antibodies against the endogenous substrate (9, chapter 4). This partly purified enzyme preparation is attached to Sepharose beads and is called solid phase carboxylase (Sp-carboxylase). The enzymatic activity of Sp-carboxylase is strictly dependent on the presence of phospholipids and in the presence of an excess of exogenous substrate (Phe-Leu-Glu-Glu-Leu), the carboxylation rate was constant for at least 3 h at 25°C (10, chapter 5, chapter 6). In this chapter we describe the results of some more detailed investigations concerning the inhibition of the Sp-carboxylase catalyzed reaction by NaCN.

MATERIALS AND METHODS

Unless indicated otherwise, all chemicals were from Merck. All buffers were made free of CO_2 by boiling and flushing with nitrogen gas.

One year old cows were treated with warfarin (Sigma) during 1 week (10 mg/kg daily) and Sp-carboxylase was prepared from the livers of these cows (chapter 4). The vitamin K-dependent incorporation of $^{14}CO_2$ was performed in reaction mixtures (0.25 ml), containing 0.1 ml Sp-carboxylase slurry (80 µg of protein), 150 mM NaCl, 50 mM Tris-acetate (pH 7.0), 2 mM Phe-Leu-Glu-Glu-Leu (Vega Fox), 2 mM dithiothreitol (Sigma), 0,2 mM EDTA, 20 µCi $NaH^{14}CO_3$ (New England Nuclear, 40 Ci/mol) and 0.2 mM vitamin K hydroquinone, which was added in the form of mixed micelles with phosphatidylcholine and Triton X-100 (chapter 6, 10). The reaction mixtures were incubated for 90 min at 25°C and the reaction was stopped by adding 2 ml icecold trichloroacetic acid (10% w/w). The supernatants were degassed at 80°C and counted. Counting was performed in a Packard Tricarb scintillation counter using Picofluor-15 (Packard) as a scintillation liquid. The vitamin K-dependent carboxylation is expressed as the amount of CO_2 (nmol) incorporated into Phe-Leu-Glu-Glu-Leu per mg of Sepharose-bound protein and was performed with preincubated Sp-carboxylase (chapter 5).

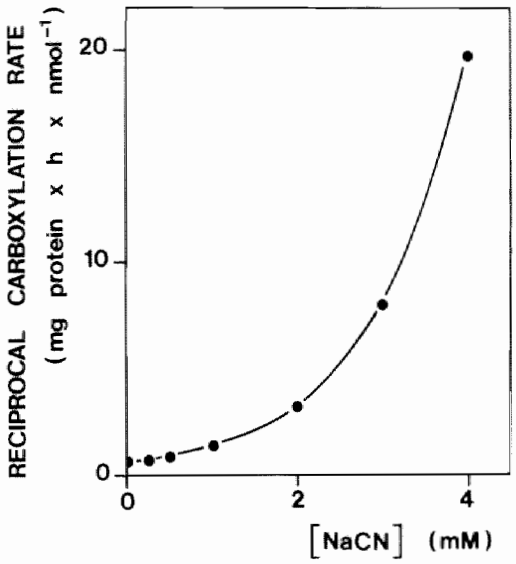
The t-butylhydroperoxide-driven carboxylation was measured under similar conditions as described for the vitamin K-dependent reaction, except for the fact that t-butylhydroperoxide (5 mM) was added instead of vitamin K hydroquinone. The t-butylhydroperoxide-dependent carboxylation is expressed as the total amount of $^{14}CO_2$ incorporated per mg of protein.

Experiments in which we checked the eventual incorporation of $^{14}CN^-$ were performed in CO_2-free buffers under similar conditions as described for the carboxylation reaction except for the fact that 100 µCi $Na^{14}CN$ (New England Nuclear, 50 Ci/mol) was added instead of $NaH^{14}CO_3$. The reaction was stopped by adding 100 mM non-labeled NaCN and non-bound label was removed by dialysis. The low molecular weight Phe-Leu-Glu-Glu-Leu (and carboxylated product) was bound to DEAE Sephadex (Pharmacia) before dialysis.

Vitamin K epoxide was determined by extracting the reaction mixtures with isoproponal/hexane and analyzing the extract with the aid of high performance liquid chromatography (chapter 2, 11).

Bovine vitamin K-dependent carboxylase was inhibited by NaCN in the solubilized microsomal fraction as well as in Sp-carboxylase. The nature of the cyanide inhibition was studied only in the more purified system. In dose-response studies in which the carboxylation reaction was performed at various NaCN concentrations, we observed a parabolic relation between the NaCN concentration and the inversed reaction rate (fig 1), indicating that the inhibition is of the non-linear type (12).

Fig. 1. The inhibition of Sp-carboxylase by NaCN.



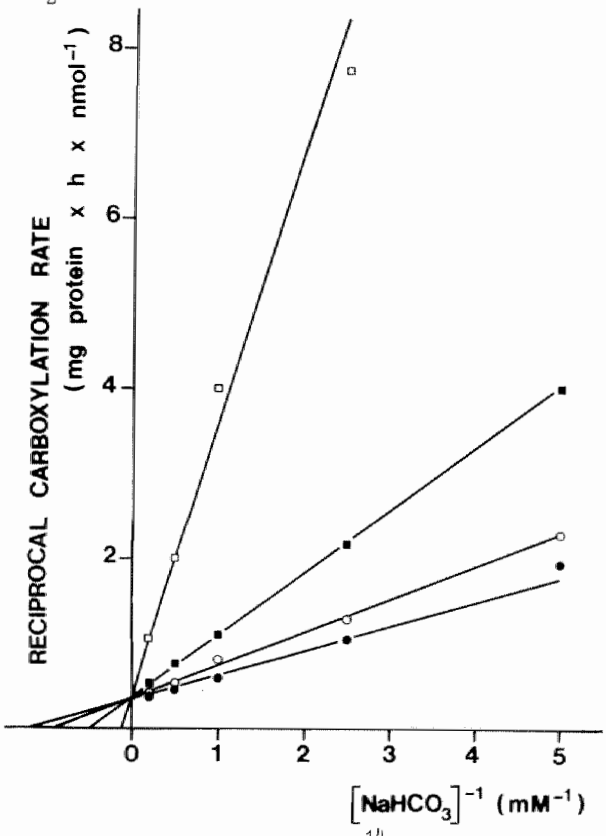
The vitamin K-dependent carboxylation was measured in the presence of NaCN as described in Materials and Methods.

Fifty percent inhibition was obtained at a cyanide concentration of 1 mM. When t-butylhydroperoxide was used as a coenzyme for Sp-carboxylase instead of vitamin K hydroquinone(chapter 5), the carboxylation reaction was 28,000 dpm per mg protein per hour, which is about 10% of its normal rate. In this system the inhibition of the carboxylation occurred at similar NaCN concentrations as in the vitamin K-dependent reaction. We also found the

cyanide inhibition to be reversible. After incubation of Sp-carboxylase with 100 mM NaCN for 1 h at 25°C, the enzyme activity could be restored up to 80% by subsequent dialysis.

In order to find out, whether the inhibition by cyanide could be counteracted by increasing the concentration of one of the participants of the carboxylation reaction, we varied the concentrations of exogenous substrate, vitamin K hydroquinone, O_2 and CO_2, whereas the cyanide concentration was kept constant. These experiments were performed at 0,25, 1 and 2 mM NaCN and it resulted, that the cyanide was competitive with CO_2 (fig 2).

Fig. 2. Lineweaver-Burk plots of the vitamin K-dependent incorporation of $^{14}CO_2$ at various NaCN concentrations.



The rate of CO_2 incorporation at a number of $NaH^{14}CO_3$ concentrations was measured in the absence of NaCN (●——●), at 0.25 mM NaCN (○——○), 1 mM NaCN (■——■) and 2 mM NaCN (□——□).

No competition was observed with one of the other reaction components.

As CO_2 is the material, incorporated in the vitamin K-dependent reaction, it might be that after adding NaCN, the inhibitor was incorporated into glutamic acid residues. Therefore we compared the incorporation of $^{14}CN^-$ and $^{14}CO_2$ by vitamin K-dependent Sp-carboxylase. Although $^{14}CO_2$ was incorporated with a rate of 250,000 dpm per h per mg protein, the incorporation of $^{14}CN^-$ could not be demonstrated.

The vitamin K-dependent carboxylation of glutamic acid residues and the epoxidation of vitamin K hydroquinone are two closely associated functions. Hence we also studied the influence of cyanide on the epoxidation reaction (table I). In order to inhibit trace amounts of reductase, which might be present in the system, we added 100 µM warfarin to these reaction mixtures. Whereas the carboxylation is completely blocked at 5 mM NaCN, no inhibition of epoxide formation was observed even at a NaCN concentration of 20 mM. This clearly demonstrated, that the formation of vitamin K epoxide is not necessarily coupled to the carboxylation reaction.

Table 1. The effect of NaCN on the vitamin K-dependent carboxylation and the vitamin K-dependent epoxidation.

The vitamin K-dependent incorporation of $^{14}CO_2$ and the formation of vitamin K epoxide were measured in standard reaction mixtures (1 ml), containing 100 µM warfarin. After incubation for 90 min at 25 C, 0.4 ml was used for determining the amount of incorporated CO_2 and 0.4 ml was taken for assesing the amount of epoxide.

NaCN concentration (mM)	$^{14}CO_2$ incorporation (nmol per mg per h)	vitamin K epoxide generation (nmol per mg per h)
0	2.5	46
2	0.4	48
20	0	53

The inhibition of carboxylase by NaCN might be explained by the binding of CN^- to a functional haem group. Therefore we resolubilized carboxylase from the solid phase with the aid of 6 M urea and 2% sodium dodecyl sulphate. Whereas under similar conditions haem could be detected

(as measured by the optical density at 415 nm) in crude microsomes (2.5 nmol per mg protein), we could not detect haem in our purified preparation, which indicates, that the amount of haem in carboxylase is less than 0.05 nmol per of mg protein.

DISCUSSION

The inhibition of vitamin K-dependent carboxylase by cyanide was found to be competitive with CO_2. It is not probable, that cyanide and CO_2 have the same or partially the same binding sites, however. In the first place, the non-linearity of the inhibition by cyanide indicates, that more than one binding site for cyanide exists on carboxylase, and that there is a cooperativity in the cyanide binding by carboxylase. On the other hand, we do not have any indication for more than one binding site for CO_2. Secondly, the electrophilic CO_2 is chemically very different from the nucleophilic CN^-. In this perspective it is not surprising that we did not find a vitamin K-dependent incorporation of CN^-.

A possible involvement of haem-iron is not substantiated by the observed type of cyanide inhibition, since in that case the inhibition would not be competitive with CO_2 but rather with more reducing agents like vitamin K hydroquinone. A second argument for the non-involvement of haem is that haem does not co-purify with carboxylase, but is removed for more than 98% from Sp-carboxylase as compared to the crude microsomal solution.

A hydroperoxide intermediate of vitamin K has been postulated as the coenzyme, required for the carboxylation event (13), and it has been shown that also t-butylhydroperoxide supports the carboxylation reaction, even in the absence of vitamin K (13,14). We observed, that the t-butylhydroperoxide-driven carboxylase co-purifies with the vitamin K-dependent enzyme and that both activities are blocked by NaCN. This supports the hypothesis that both, the t-butylhydroperoxide-driven carboxylation and the vitamin K-dependent carboxylation are accomplished by the same enzyme.

It has been postulated by several authors, that the vitamin K-dependent carboxylation is coupled to the epoxidation of vitamin K hydroquinone. Carboxylation without epoxidation has never been demonstrated, and inhibitors of the epoxidation reaction (e.g. Chloro-K and peroxidases) also

inhibit the carboxylation reaction (7,15). On the other hand, the epoxidation event seems not necessarily to be coupled to the carboxylation event because, on a molar basis, the amount of epoxide formed always exceeds the amount of incorporated CO_2 (3). Moreover, epoxidation has even been demonstrated in the absence of CO_2 (15). This uncoupling of the two reactions is also illustrated by the fact that cyanide blocks the carboxylation reaction without a simultaneous inhibition of the epoxide formation. Cyanide might therefore be helpful when the epoxidation event has to be studied in the absence of carboxylation. The question whether epoxidation and carboxylation are both catalyzed by the same enzyme remains to be answered, however.

REFERENCES

1. Johnson, R.C. (1980) Methods Enzymol. 67, 165-180
2. Gallop, P.M., Lian, J.B. and Hauschka, P.V. (1980) New Engl. J. Med. 302, 1460-1466
3. Suttie, J.W. (1980) C.R.C. Crit. Rev. Biochem. 8, 191-223
4. Suttie, J.W., Geweke, L.O., Martin, S.L. and Willingham, A.K. (1980) FEBS Lett. 109, 267-270
5. Hall, A.L., Bettger, W.J. and Olson, R.E. (1981) Fed. Proc. 40, 3650
6. Larsen, A.E., McTigue, J.J. and Suttie, J.W. (1980) in: Vitamin K metabolism and vitamin K dependent proteins (J.W. Suttie ed.) pp 413-421, University Park Press, Baltimore, M.D.
7. Suttie, J.W., Larson, A.E., Canfield, L.M. and Carlisle, T.L. (1978) Fed. Proc. 37, 2605-2609
8. Larson, A.E., Witlon, D.S. and Suttie, J.W. (1979) Fed. Proc. 38, 3410
9. De Metz, M., Vermeer, C., Soute, B.A.M., van Scharrenburg, G.J.M., Slotboom, A.J. and Hemker, H.C. (1981) FEBS Lett. 123, 215-218
10. De Metz, M., Vermeer, C., Soute, B.A.M. and Hemker, H.C. (1981), J. Biol. Chem., 256, 10843-10846
11. Vermeer, C., Soute, B.A.M., de Metz, M. and Hemker, H.C. (1982) Biochim. Biophys. Acta 714, 361-365

12. Cleland, W.W. (1970) in: The Enzymes, Vol II, 3rd ed., (P.D. Boyer, ed.), Acadamic Press, New York, pp 1-65
13. Larson, A.E. and Suttie, J.W. (1978) Proc. Natl. Acad. Sci. USA 75, 5413-5416
14. Esnouf, M.P., Burgess, A.I., Walter, S.J., Green, M.R., Hill, M.A.O. and Okolow-Zubkowska, M.J. (1980) in: Vitamin K metabolism and vitamin K dependent proteins (J.W. Suttie, ed.) pp 422-432, University Park Press, Baltimore, MD
15. Bell, R.G. (1978) Fed. Proc. 37, 2599-2604
16. Sadowski, J.A., Schnoes, H.K. and Suttie, J.W. (1977) Biochemistry 16, 3856-3863

CHAPTER 8

STUDIES ON THE MECHANISM OF THE VITAMIN K-DEPENDENT CARBOXYLATION REACTION; CARBOXYLATION WITHOUT THE CONCURRENT FORMATION OF VITAMIN K 2,3-EPOXIDE.

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SUMMARY

In addition to the three known forms of vitamin K (vitamin K quinone, vitamin K hydroquinone and vitamin K epoxide), a fourth metabolite, hydroxyvitamin K, was found in reaction mixtures containing a vitamin K-dependent carboxylating enzyme system. When sulphite was added to such reaction mixtures, the formation of hydroxyvitamin K was substantially enhanced, whereas no epoxide was formed anymore. The vitamin K-dependent carboxylation was stimulated at these sulphite concentrations. Vitamin K hydroquinone could be replaced by t-butylhydroperoxide and also under these conditions the carboxylation was enhanced by sulphite. In the presence of peroxidase the carboxylation reaction was blocked, whereas hydroxyvitamin K could still be detected in the reaction mixtures, even in the absence of sulphite. These observations lead us to the hypothesis, that the carboxylation of glutamic acid residues is coupled to the heterolytic cleavage of a peroxyde bond with the concurrent formation of vitamin epoxide.

INTRODUCTION

Vitamin K-dependent carboxylase has been obtained from various tissues and from a number of species (1-4). The only carboxylating enzyme system that could be purified to a reasonable extent, is bovine liver carboxylase (5,6). The purification occurs with the aid of antibodies to blood clotting factor X, which are cross-linked to Sepharose. The antibodies are able to extract complexes of a factor X-precursor and carboxylase from a crude detergent-solubilized microsomal preparation. The solid-phase carboxylase thus formed (Sp-carboxylase) was analyzed and it could be demonstrated, that the enzyme is constituted of 30% (w/w) phosphatidylcholine and 70% (w/w) proteins (chapter 6). In this chapter we describe a) the extraction and analysis of vitamin K from Sp-carboxylase during the carboxylation reaction and b) the detection of a number of intermediate forms to which vitamin K is converted during this reaction.

MATERIALS AND METHODS

Materials. Triton X-100, vitamin K_1, warfarin, horse-radish peroxidase and dithiothreitol were obtained from Sigma, $NaH^{14}CO_3$ (40 mCi/mmol) and Aquasol-2 from New England Nuclear. ^3H-labeled vitamin K_1 was a kind gift of Hoffmann-La Roche. The synthetic pentapeptide Phe-Leu-Glu-Glu-Leu was purchased from Vega Fox. All other chemicals were from Merck. Vitamin K epoxide and vitamin K hydroquinone were prepared as described earlier (8)

Methods. Solid phase carboxylase (Sp-carboxylase) was prepared from the livers of warfarin-treated cows (chapter 4, 6) The incorporation of $^{14}CO_2$ was measured as described by de Metz et al. (chapter 5, 7). In order to inhibit traces of reductase, which might be present in Sp-carboxylase, we added 20 µM warfarin to all reaction mixtures. All data are given as the sum of the carboxylation of the endogenous and exogenous substrate.

The various forms of vitamin K were extracted from carboxylating reaction mixtures (1 ml) by adding 10 ml hexane and 15 ml isopropanol, followed by shaking the mixtures for 5 min. Subsequently 5 ml water was added and the lower phase was discarded. The solvent of the upper phase was evaporated on a rotavapor and the remaining vitamin K metabolites were dissolved in 0.2 ml methanol and analyzed by means of high performance liquid chromatography on a Spectraphysics SP 8000 liquid chromatograph, using a CP Spher C 18 column (Chrompack, 25 x 0.46 cm) in methanol. The flow rate was 2 ml per min and the effluent was monitored at 254 nm. The various forms of vitamin K were well separated and the area under each peak was recorded automatically and corrected for the relative absorbance at the detection wavelength, using ^3H-labeled vitamin K as a standard.

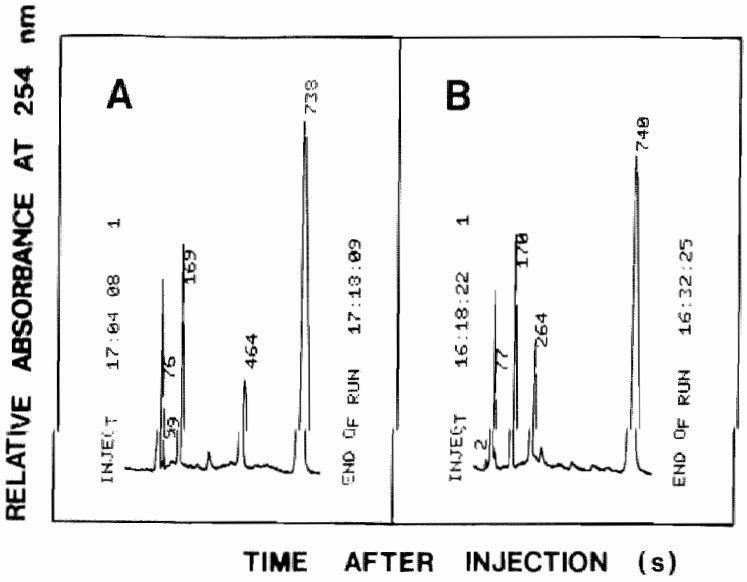
Field desorption mass spectra (9) of samples from the various peaks were taken using a Varian Mat 711 double focusing mass spectrometer with a combined electron impact/field ionization/field desorption ion source and coupled to a spectro system MAT 100 data acquisition unit. 10 µm-tungsten wire FD emitters containing carbon microneedles with an average length of 30 µm were used. The samples were dissolved in methanol and loaded onto the emitters with the dipping technique. The ion source was 30°C. During the high resolution field desorption mass spectroscopy measurements, a resolving power of 9000 (10% valley definition) was used.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was performed according to Laemmli (10) and protein concentrations were determined as described by Lowry and coworkers (11). CO_2 concentrations in the reaction mixtures were assessed as described by Rose (12)

RESULTS

Carboxylating reaction mixtures, containing Sp-carboxylase and vitamin K hydroquinone, were incubated and after 5 h vitamin K was extracted with isopropanol/hexane and analyzed by high performance liquid chromatography (fig 1A).

Fig. 1. The detection of various vitamin K metabolites.



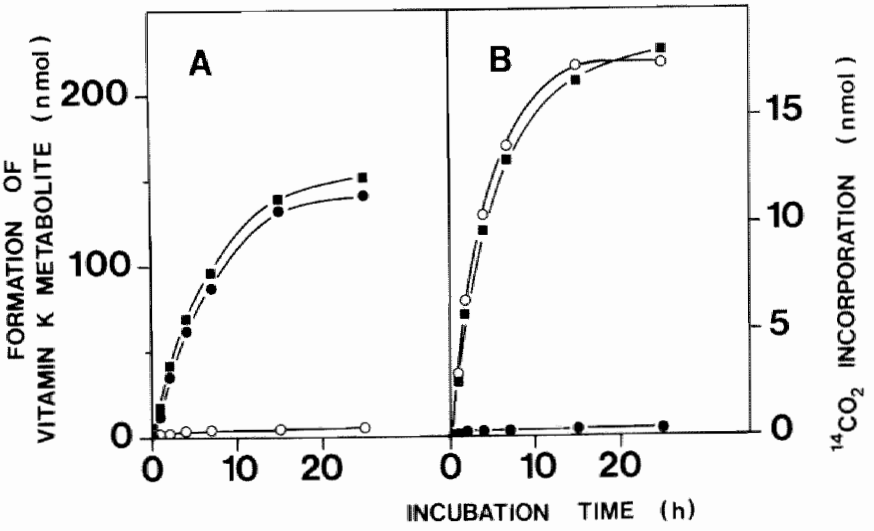
A: separation profile of vitamin K metabolites after extraction of a carboxylating reaction mixture (containing 2 mM Phe-Leu-Glu-Glu-Leu), which had been incubated for 5 h. The peaks eluting after 169, 464 and 738 s represent vitamin K hydroquinone, vitamin K epoxide and vitamin K, respectively.

B: separation profile of vitamin K metabolites after extraction of a similar carboxylating reaction mixture which had been incubated for 5 h in the presence of 60 mM Na_2SO_3. The peak eluting after 264 s represents hydroxyvitamin K.

In the purified system the carboxylation reaction proceeds with the simultaneous formation of vitamin K epoxide, in the same way as in the crude microsomal system. The high peak of normal vitamin K was the result of a rapid non-enzymatic oxidation of vitamin K hydroquinone.

When we added sulphite (a disodium sulphite solution, adjusted to pH 7.4 with 1 M HCl) to similar reaction mixtures as described above, the carboxylation reaction was stimulated 1.5 fold, whereas no vitamin K epoxide was formed anymore. Instead of the epoxide, a new peak was monitored (fig 1B), collected and analyzed by mass spectrometry. It resulted, that the newly formed peak was a hydroxylated vitamin K.
The observed molecular mass was 468.3605, which is close to the calculated value (468.3603). The same results were obtained when benzenesulphinate was used in stead of sulphite. Moreover, when the number of carboxylation events was reduced by omitting the pentapeptide, the formation of hydro-

Fig. 2. Time course of the formation of vitamin K epoxide, hydroxy-vitamin K and the $^{14}CO_2$ incorporation.



Time course of the formation of vitamin K epoxide (●——●), hydroxyvitamin K (○——○) and the $^{14}CO_2$ incorporation (■——■). The latter is given as the sum of the carboxylation of the endogenous and exogenous substrate. A: in the absence of sulpite, B: in the presence of 60 mM disodium sulphite.

xylated vitamin K was reduced proportionally. Control experiments showed, that hydroxylated vitamin K could not be generated from vitamin K epoxide under the experimental conditions. The time-course of the formation of hydroxyvitamin K was similar to that of vitamin K epoxide (fig 2). When calculated on a molar base, the rate of formation of both forms of vitamin K was 10 fold higher than the rate of CO_2 incorporation.

When we added horse-radish peroxidase or Fe^{++} (instead of sulphite) to carboxylating reaction mixtures, we observed that also in this case the formation of vitamin K epoxide was blocked and again hydroxyvitamin K could be detected in the reaction mixtures (table 1). The main difference between the influence of sulphite and that of peroxidase (or Fe^{++}) was that the carboxylation reaction was stimulated by sulphite, but that it was strongly inhibited by peroxidase.

Table 1. The effect of peroxidase and Fe^{++} on Sp-carboxylase mediated reactions.

Enzymes added	formation of vitamin K epoxide	formation of hydroxy-vitamin K	CO_2 incorporation
None	1	0.5	0
Sp-carboxylase (80 µg)	35	1.5	2.8
Sp-carboxylase (80 µg) and peroxidase (10 µg)	1	8	0.06
Sp-carboxylase (80 µg) and Fe^{++} (0.1 mM)	0	4	0
Peroxidase (10 µg)	0	3.5	0

Reaction mixtures (1 ml) containing 2 mM dithiothreitol, 0.1 mM vitamin K hydroquinone 2 mM Phe-Leu-Glu-Glu-Leu, 1 mM $NaH^{14}CO_3$, 1 mM phenol and enzymes as indicated were incubated and analyzed as described in the text. When Fe^{++} was added, EDTA was omitted from the reaction mixture. Control experiments in which peroxidase was inactivated, either by omitting phenol or by adding 50 mM sodiumazide gave similar results as in the absence of peroxidase. The amount of incorporated CO_2 was calculated after determining the concentrations of labeled (1 mM) and unlabeled (1.2 mM) CO_2 in the reaction mixtures. All data are expressed as nmol per mg carboxylase per h.

We concluded therefore, that the hydroxyvitamin K was formed from a vitamin K peroxide and that this peroxide is destroyed by peroxidase or Fe^{++} before it can participate in the carboxylation reaction. On the other hand, the reduction of the peroxide by sulphite seems to occur during the carboxylation reaction in such a way, that this reaction is even slightly stimulated. The difference between the effect of sulphite and that of peroxidase became even more evident in control experiments, in which Sp-carboxylase was omitted. Whereas the formation of hydroxyvitamin K by sulphite was strictly dependent on the presence of carboxylase, the peroxidase-catalyzed reaction also proceeded in the absence of carboxylase. The latter observation strongly suggests, that the formation of vitamin K peroxides occurs spontaneously under normal incubation conditions.

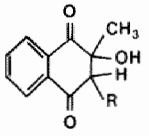
In order to make sure, that the cleavage of the peroxide bond is the step, required for the carboxylation reaction, we replaced vitamin K hydroquinone by t-butylhydroperoxide. The latter is known to stimulate carboxylase in the crude microsomal system from rat liver (13). It resulted that like vitamin K hydroquinone, t-butylhydroperoxide is able to serve as a co-enzyme for Sp-carboxylase. The reaction was influenced by a number of inhibitors and stimulating agents in a similar way as was the vitamin K-dependent reaction (chapter 5, chapter 7) and after acid hydrolysis (24 h) of the reaction product the incorporated label was reduced by 50 %. Also in the t-butylhydroperoxide-driven reaction sulphite stimulates the carboxylation (about 3 fold). So it seems that sulphite is directly involved in the cleavage of the peroxide bond and that the formation of an epoxide is not an absolute requirement for carboxylase activity.

DISCUSSION

During the last few years a number of authors have speculated about the possiblity that the formation of vitamin K epoxide was coupled to the vitamin K-dependent carboxylation reaction (14). Because it appeared to be very difficult to purify carboxylase to homogenity, it could not be demonstrated unequivocally, that both activities were due to the same enzyme. Until now, Sp-carboxylase is the most purified enzyme system, described in the literature, in which the vitamin K-dependent carboxylation reaction can

take place. Since even in this purified system the incorporation of CO_2 always occurred with the simultaneous production of vitamin K epoxide, we assume that both, the carboxylated glutamic acid residues and the vitamin K epoxide are the products of the same reaction. Any reaction mechanism that is proposed should therefore explain the formation of both reaction products.

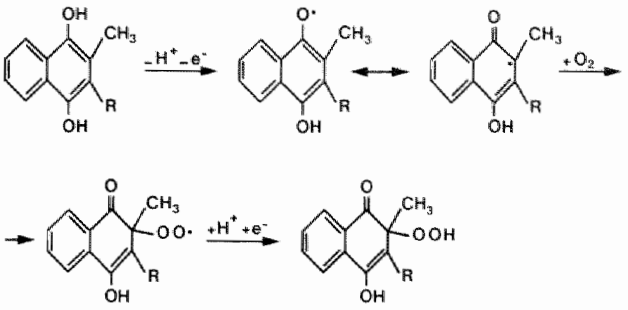
Because peroxidase strongly inhibits the carboxylation reaction, it seems plausible, that the formation of a vitamin K peroxide precedes this carboxylation reaction. This hypothesis was formulated for the first time by Larson and Suttie (15), who suggested the formation of vitamin K hydroperoxide. The formation of such a hydroperoxide is plausible because naphtoquinones are known to readily form radicals. Once the unpaired electron has been generated , it may migrate through the conjugated ring system and initiate the oxidation of a double bond. We could demonstrate the formation of hydroxyvitamin K by the action of peroxidase, which is compatable with this theory, because the hydroxyvitamin K may be formed from vitamin K hydroperoxide. This would imply that the hydroxyl group is located at the 2 or at the 3 position.



2-hydroxyvitamin K

With the techniques used in these experiments we were not able to distinguish between 2-hydroxyvitamin K and 3-hydroxyvitamin K. Further studies by which we can discriminate between these two forms are in current progress. Since hydroxyvitamin K was also formed by peroxidase in the absence of carboxylase, the latter is thus not necessarily involved in the formation of the hydroperoxide. Therefore vitamin K hydroperoxide may be regarded as the real coenzyme for carboxylase. Since in general hydroquinones rapidly form radicals, and since radical scavengers are inhibitors of carboxylase (16), it seems that the hydroperoxide is formed according to equation 1.

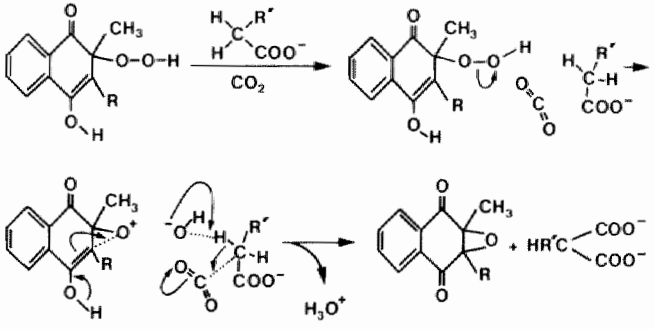
(equation 1)



We do not think, that the hydroperoxyde is formed via oxygen radicals ('OH, singlet O_2 or O_2^-), because we have never been able to demonstrate any inhibition of carboxylase by inhibitors of these forms of oxygen (chapter 5). Similar results were obtained by Larson (16) who did not find any stimulation of the carboxylation reaction by these oxygen radicals, moreover.

The role of carboxylase may be that it couples the cleavage of the peroxide bond to the carboxylation of a glutamic acid residue. In the absence of sulphite, the products of this reaction are vitamin K epoxide and a gamma-carboxy glutamic acid residue. We propose, that the removal of a gamma hydrogen atom from the glutamic acid residue and the addition of carbon dioxide occur in a concerted reaction (equation 2).

(equation 2)



This implies that the peroxide bond is cleaved in a heterolytic way giving rise to a positive charge on the vitamin K-bound oxygen and OH^-. The enzyme-bound OH^- may then be used to remove a gamma-hydrogen atom from the glutamic acid residue and the resulting carbanion will react with CO_2. When sulphite is present in the reaction mixture, it might weaken the peroxide bond by its reducing character thus stimulating the electron shift and the carboxyl- ation reaction. At the same time the vitamin K-bound oxygen is reduced via a two-electron transfer (sulphite is a two-electron donor) and subsequently protonated to hydroxyvitamin K.

Alternatively, hydroxyvitamin K might also be formed by an interaction of sulphite with vitamin K epoxide. The stimulation of the carboxylation reaction would then be explained by a rapid consumption of one of the reaction products (vitamin K epoxide) by sulphite. This possiblility is less likely, because a) we have never been able to demonstrate the formation of hydroxyvitamin K from vitamin K epoxide and sulphite and b) in the t-butylhydroperoxide-driven reaction no epoxide can be formed and yet sulphite stimulates this reaction to a similar extent as it stimulates the vitamin K hydroquinone-driven reaction. The mechanism of the t-butyl-hydroperoxide-driven reaction may also start with the heterolytic cleavage of the peroxide bond. The remaining positively charged t-butyloxide will then either be reduced by the dithiothreitol present in the reaction mixture, or it will undergo a methyl shift and a subsequent hydrolysis to acetone and methanol.

Since on a molar basis more vitamin K epoxide and hydroxyvitamin K are formed than carbon dioxide is incorporated, we assume that the epoxide formation does not always lead to carboxylation. This is in agreement with experiments of Friedman (17), who demonstrated an exchange of ^3H-labeled gamma hydrogen atoms. This exchange was vitamin K-dependent and occurred with a 10 times higher reaction rate than the incorporation of CO_2. We suggest, that in the uncoupled reaction a proton takes the place of CO_2, thus giving rise to the exchange of the gamma hydrogen with water and the formation of vitamin K epoxide.

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REFERENCES

1. Suttie, J.W., Lehrman, S.R., Geweke, L.O., Hageman, J.M. and Rich, D.H. (1979) Biochem. Biophys. Res. Comm. 86, 500-507
2. Bell, R.G. (1980) Arch. Biochem. Biophys. 203, 58-64
3. Hauschka, P.V., Friedman, P.A., Traverso, H.P. and Gallop, P.M. (1976) Biochem. Biophys. Res. Comm. 71, 1207-1213
4. Lian, J.B. and Friedman, P.A. (1978) 253, 6623-6626
5. Canfield, L.M. Sinsky, T.A. and Suttie, J.W. (1980) Arch. Biochem. Biophys. 202, 515-524
6. De Metz, M., Vermeer, C., Soute, B.A.M. and Hemker, H.C. (1981) FEBS Lett. 123, 215-218
7. De Metz, M., Vermeer, C., Soute, B.A.M. and Hemker, H.C. (1981) J. Biol. Chem. 256, 10843-10846
8. De Metz, M., Vermeer, C., Soute, B.A.M. and Hemker, H.C. (1980) in: Vitamin K metabolism and vitamin K dependent proteins (Suttie, J.W. e.d.), pp. 560-570, University Park Press, Baltimore
9. Schulten, H.R. (1979) Intern.J.Mass Spectrom.Ion.Phys. 32, 97-283
10. Laemmli, U.K. (1970) Nature 227, 680-685
11. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275
12. Rose, J.G. (1976) Anal. Biochem. 76, 358-360
13. Suttie, J.W., Larson, A.E., Canfield, L.M. and Carlisle, T.L. (1978) Fed. Proc. 37, 2605-2609
14. Suttie, J.W., Geweke, L.O., Martin, S.L. and Willingham, A.K. (1980) FEBS Lett. 109, 267-270
15. Larson, A.E. and Suttie, J.W. (1978) Proc. Natl. Acad. Sci. USA 75, 5413-5416
16. Larson, A.E., McTigue, J.J. and Suttie, J.W. (1980) in: Vitamin K metabolism and vitamin K dependent proteins, (Suttie, J.W., ed.) pp.413-421, University Park Press, Baltimore,
17. Friedman, P.A.(1980) in Vitamin K metabolism and vitamin K dependent proteins, (Suttie,J.W.,ed.) pp.401-407, University Park Press,Baltimore

CHAPTER 9

CONCLUSIONS AND SUMMARY

Vitamin K is involved in the carboxylation of a great number of proteins (amongst which four coagulation factors). By this reaction some of the glutamic acid residues in the polypeptide chain are converted into γ-carboxyglutamic acid residues. In this thesis we describe some characteristics of the vitamin K-dependent carboxylation reaction in cow liver.

When cows are fed with vitamin K-antagonists, the vitamin K-dependent reactions are blocked, which results in the appearance of descarboxyfactors in the blood and in an accumulation (50-100 fold) of clotting factor precursors in the liver (chapter 2 and 4). In our in vitro system these precursors serve as an endogenous substrate for carboxylase and they mainly consist of precursors of factor X (60%) and prothrombin (25%). The hepatic factor X precursor is a single chain protein. Since plasma factor X is a two-chain molecule, this observation strongly suggests, that factor X is synthesized as a single polypeptide chain, which is cleaved during the last stage of its maturation. The almost complete identification of the reaction products of carboxylase could only be obtained in the bovine system. In rat carboxylase for instance, only 25% of the endogenous substrate was identified (1).

In chapter 3 we have shown that descarboxyprothrombin and descarboxy-factor X, purified from blood, hardly affect the in vitro carboxylation reaction at plasma concentrations. It could be established that descarboxy-prothrombin precursors are substrate molecules albeit with a high K_m. A proteolytic degradation product of descarboxyprothrombin (the amino acids 13-29) was a far better substrate than the intact molecule. The K_m for carboxylase was lowered at least 1000 fold after degradation with subtilisin. The minimal requirement for a good substrate has not been established and it is unknown which other descarboxyfactors contain the intrinsic information to be a good substrate for the carboxylase preparation used. It is not unlikely, however, that at least the clotting factor X and prothrombin are carboxylated by the same enzyme since carboxylase purified with the aid of immunoadsorption to antifactor X, is

still active in the carboxylation of descarboxyprothrombin and its degradation products. The specificity of carboxylase preparations from various organs is a subject of current studies.

The development of low molecular weight substrates for the vitamin K-dependent carboxylase (2,3) has rendered the possibility to study the carboxylation reaction without being dependent on the presence of endogenous substrates. With one of the substrates (Phe-Leu-Glu-Glu-Leu) it could be demonstrated that warfarin treatment of cows does not affect the level and the properties of hepatic vitamin K-dependent carboxylase (chapter 2).

When measured in the microsomal fraction, the carboxylation reaction can be driven by vitamin K quinone, vitamin K hydroquinone as well as by vitamin K epoxide. The highest carboxylation rate is obtained with vitamin K hydroquinone. Vitamin K hydroquinone is believed to be the form which is driving the carboxylation event (3,4) and this implies that vitamin K is first reduced by vitamin K reductase before entering the carboxylating enzyme system. In the presence of vitamin K hydroquinone carboxylase may function independent of the reductase, when the latter enzyme is blocked by warfarin. Vitamin K epoxide is formed in parallel with the carboxylation reaction and can be reduced by vitamin K epoxide reductase. In our system the reduction of vitamin K epoxide (and vitamin K) could be uncoupled from the carboxylation by using an enzyme preparation without any substrate for the carboxylase. So both carboxylase and reductase may function independently.

Nevertheless it seems probable that carboxylase and reductase are associated within the microsomal membrane (chapter 2). Furthermore we concluded that an intimate link exists between the endogenous substrate and the enzyme, because the complex is retained on a column on which antibodies against the endogenous substrate are present (chapter 4). Also, we showed (chapter 6) that phospholipid (primarily phosphatidylcholine) is an essential constituent of the vitamin K-depedent carboxylase. Therefore we postulate the existance of an enzyme complex of carboxylase, reductase, endogenous substrate (if present) and phospholipid. The phospholipid moiety might function in the transport and/or storage of various forms of vitamin K e.g. vitamin K hydroquinone produced by the reductase.

The carboxylase is a constituent of the rough endoplasmic reticulum

(5,6), which can be extracted therefrom with a number of detergents. After solubilization a considerable purification could be achieved with immunoadsorption onto antibodies against the endogenous substrate (chapter 4). No other satisfactory purification methods have been published until now. In a number of cases the difficulties with the purification might be explained by a loss of the enzyme activity caused by the separation of phospholipid and proteins in the presence of detergents. At least in the bovine system phospholipids are essential for carboxylase activity (chapter 6).

As has been pointed out in chapter 5 the semi-purified carboxylase, after elution from the solid phase, is a high molecular weight moiety consisting of phospholipid and various proteins. For further purification carboxylase has to be re-solubilized and until now this has not been achieved without loosing the main part of the enzyme activity. We have no indications that cofactors other than vitamin K (e.g. heme) are involved in the carboxylation reaction (chapter 7).

Sp-carboxylase is a useful enzyme preparation for studying the vitamin K-dependent carboxylation reaction. Most properties are similar to those of other known carboxylase preparations and the purification procedure is simple. The attachement to the Sepahrose beads is advantageous when a quick washing procedure is needed e.g. after the phospholipase treatment (chapter 6). Sp-carboxylase differs from other preparations in several ways: it is more pure, it does contain a low amount of vitamin K-reductase, the carboxylation rate of Phe-Leu-Glu-Glu-Leu is linear for a longer period, the carboxylation reaction has a higher optimal temperature and the reaction is inhibited by low detergent concentrations.

It is generally believed that the vitamin K-dependent carboxylation reaction is coupled to the epoxidation of vitamin K hydroquinone (see chapter 1). Radicals and a hydroperoxide intermediate might be involved in this epoxide formation. The idea that hydroperoxide vitamin K is involved was substantiated by the peroxidase inhibition and the carboxylation with t-butylhydroperoxide present instead of vitamin K hydroquinone (7). The t-butylhydroperoxide driven carboxylation was small however and might be effected by an impurity in the carboxylase preparation (8). With the semi purified Sp-carboxylase we could clearly demonstrate that the properties of the t-butylhydroperoxide driven carboxylation were similar to the properties of the vitamin K-dependent carboxylation (chapter 5).

The epoxidation is not absolutely coupled to the carboxylation (4). In most systems more vitamin K epoxide is formed than CO_2 incorporated (on a molar basis). With CN^- the carboxylation could even be completely blocked without blocking the epoxidation (chapter 7, ref. 9).

In chapter 8 we showed that carboxylation can occur without the concurrent formation of vitamin K epoxide. In the presence of sulphite a hydroxyvitamin K was formed instead of the epoxide. We concluded that the hydroxyvitamin K (and vitamin K epoxide when no sulphite is present) was formed from a hydroperoxide intermediate after a heterolytic fission of the peroxide bond. A the same time a carbanion is formed which can be carboxylated easily. Obviously the proposed mechanism has to be verified in a purified system.

REFERENCES

1. Willingham, A.K., Martin, S.L., Graves, C.B., Graban, G.G. and Munns, T.W. (1980) in Vitamin K Metabolism and Vitamin K dependent Proteins, (Suttie, J.W. ed.), University Park Press, Baltimore, 533
2. Houser, R.M., Carey, D.J., Dus, K.M., Marshall, G.R. and Olson, R.F. (1977) FEBS Lett. 75, 226
3. Suttie, J.W. Lehrman, S.R., Geweke, L.O., Hageman, J.H. and Rich, D.M. (1979) Biochem.Biophys.Res.Commun. 86, 500
4. Suttie, J.W. (1980) C.R.C. Crit.Rev.Biochem. 8, 191
5. Helgeland, L. (1977) Biochim.Biophys.Acta 499, 181
6. Carlisle, T.L. and Suttie, J.W. (1980) Biochemistry 1,1161
7. Larson,A.E. and Suttie, J.W. (1978) Proc.Natl.Ac.Sci.U.S.A. 75, 5413
8. Hall,A.L., Turner,P.M., Dunkle,B.F., Wing,D.A. and Olson,R.E. (1980) in Vitamin K Metabolism and Vitamin K dependent Proteins (Suttie, J.W. ed.),University Park Press, Baltimore, 433
9. Larson, A.E., Friedman, P.A. and Suttie, J.W. (1981) J.Biol.Chem. 256, 11032

SAMENVATTING

Vitamine K is nodig bij de carboxylering van een groot aantal eiwitten, waaronder verschillende stollingsfaktoren. Bij deze carboxyleringsreactie worden enkele glutaminezuur (glu) resten omgezet in γ-carboxyglutaminezuur (gla) resten. Na het toedienen van vitamine K antagonisten (hydroxycoumarinederivaten) zakt het plasma niveau van de vitamine K--afhankelijke stollingsfactoren en neemt de kans op ongewenste stolselvorming af. De orale antistollingstherapie met coumarinederivaten neemt dan ook een belangrijke plaats in bij de bestrijding en preventie van thrombose. In dit proefschrift wordt het onderzoek naar de vitamine K-afhankelijke carboxylering in runderlever beschreven.

Na het toedienen van vitamine K-antagonisten worden de vitamine K-afhankelijke reacties geremd. Descarboxyfactoren (stollingsfactoren met glu resten in plaats van gla resten) verschijnen dan in het bloed en precursors van de stollingsfactoren hopen zich op in de lever (hoofdstuk 2 en 4). In het door ons ontwikkelde in vitro systeem kunnen deze precursors vitamine K-afhankelijk gecarboxyleerd worden. In hoofdstuk 4 is aangetoond dat het grootste geddelte van de carboxyleerbare eiwitten uit precursors van factor X (ca. 60%) en prothrombine (ca. 25%) bestaat. De factor X precursor uit de lever bleek uit één eiwitketen te bestaan, terwijl het factor X uit plasma uit twee eiwitketens bestaat.

In hoofdstuk 3 hebben we aangetoond dat descarboxyprothrombine, geisoleerd uit runderbloed, substraat is voor de vitamine K-afhankelijke carboxylase uit runder- en rattelever. De affiniteit van descarboxyprothrombine voor het carboxylase is echter bijzonder laag (K_m 0.3 - 0.4 mM). Door proteolytische afbraak van descarboxyprothrombine met subtilisine Carlsberg werd een fragment verkregen met een veel hogere affiniteit voor het carboxylase (K_m = 0.001 - 0.003 mM). Het fragment bestond uit de aminozuren 13-29 van descarboxyprothrombine.

In hoofdstuk 2 wordt de vitamine K-afhankelijke carboxylase uit de lever van normale en geantistolde koeien vergeleken. Behalve in de hoeveelheid precursors, die substraat zijn voor carboxylase, werd geen verschil gevonden tussen de beide preparaten. Tevens wordt in dit hoofdstuk aangetoond dat de vitamine K-afhankelijke carboxylering en de reductie van vitamine K onafhankelijk van elkaar kunnen verlopen; de enzymen die deze

reakties katalyseren (carboxylase en reductase) zijn echter wel nauw met elkaar verbonden.

In hoofdstuk 4 wordt de zuivering van carboxylase uit gesolubilizeerde levermicrosomen beschreven. Wij maakten gebruik van het feit dat de factor X precursors, die substraat zijn voor het carboxylase, gecomplexeerd zijn met het carboxylase. Met behulp van immunospecifieke adsorptie aan antilichamen tegen de factor X precursors werd (uitgaande van de microsomale fractie) een 100-voudige zuivering verkregen. Dit gedeeltelijk gezuiverde preparaat is gebonden aan Sepharose deeltjes en wordt daarom Sp-carboxylase (Solid phase carboxylase) genoemd.

Enkele eigenschappen van Sp-carboxylase worden beschreven in hoofdstuk 5 en 6. De belangrijkste eigenschappen zijn vergelijkbaar met die van een ongezuiverd preparaat. Het Sp-carboxylase bevat nog verschillende eiwitcomponenten, waarvan twee vitamine K binden. Voorts bestaat het preparaat voor ca. 30% uit phospholipiden (voornamelijk phosphatidylcholine). Het preparaat kon met behulp van phospholipases of detergentia phospholipiden-vrij gemaakt worden. Reconstitutie tot ruim 80% van de oorspronkelijke activiteit was mogelijk door het toevoegen van micellen, die phosphatidylcholine en cholaat bevatten.

In hoofdstuk 7 wordt de remming van de vitamine K-afhankelijke carboxylering door cyanide beschreven. Het bleek een niet-lineaire remming te zijn, competitief met CO_2. De epoxidering van vitamine K hydroquinone werd niet geremd door cyanide. De remming door cyanide bleek geen argument voor de deelname van een haem groep aan de vitamine K-afhankelijke carboxyleringsreaktie. Haem kon bovendien niet aangetoond worden in Sp-carboxylase.

In hoofdstuk 8 tenslotte wordt beschreven dat de vitamine K afhankelijke carboxyleringsreaktie gekoppeld is aan de vorming van vitamine K-epoxide. In aanwezigheid van sulfiet kan de carboxyleringsreaktie echter gekoppeld worden aan de vorming van hydroxy vitamine K. De carboxyleringsreaktie werd tevens gestimuleerd in aanwezigheid van sulfiet, zowel de vitamine K-afhankelijke als ook de t-butylhydroperoxide afhankelijke carboxylering. In aanwezigheid van peroxidases werd de carboxyleringsreaktie en de vorming van vitamine K epoxide geremd, terwijl hydroxyvitamine K wel gevormd werd (ook in afwezigheid van sulfiet). Deze bevindingen leidden tot de hypothese dat de carboxylering van glutaminezuur resten gekoppeld is aan een heterolytische splitsing van een peroxide binding met de gelijktijdige vorming van vitamine K epoxide.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 14 juli 1950 te Den Haag geboren. In 1969 behaalde hij het diploma gymnasium B aan het Vossius gymnasium te Amsterdam en in datzelfde jaar begon hij met de studie scheikunde aan de Universiteit van Amsterdam. Het doctoraal examen met als hoofdvak biochemie werd in 1977 afgelegd. Van juli 1977 tot maart 1978 werkte hij als wetenschappelijk medewerker op het B.C.P.Jansen instituut te Amsterdam. Sinds april 1978 is hij als wetenschappelijk medewerker verbonden aan de capaciteitsgroep Biochemie van de Rijksuniversiteit Limburg te Maastricht. Bij deze groep werd onder leiding van dr. C.Vermeer en prof. dr. H.C.Hemker het in dit proefschrift beschreven onderzoek verricht.